UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Non canonical role of GAPDH in neutrophil migration and chronic skin inflammation

Función no canónica de GAPDH en la migración de los neutrófilos y la inflamación crónica de la piel

D. Joaquín Cantón Sandoval

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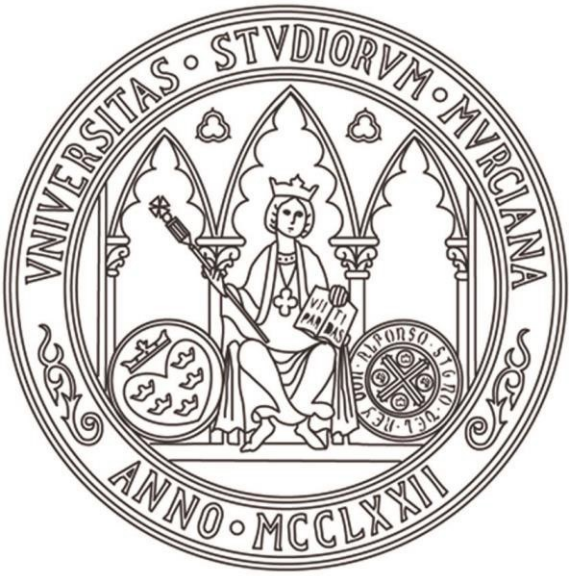

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DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD
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doctorando del Programa de Doctorado en

Programa de Doctorado en Biología Molecular y Biotecnología

de la Escuela Internacional de Doctorado de la Universidad Murcia, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

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ABSTRACT

Neutrophils are the most abundant leukocytes and have an important role in tissue homeostasis and disease. Neutrophil migration and its role during chronic inflammation have been recently the focus of huge interest due to its tremendous therapeutical potential. The zebrafish has become an excellent model for the study of inflammation and neutrophil biology with several zebrafish mutant lines allowing the study of chronic inflammation. Spint1a-deficient zebrafish larvae is a widely used model of chronic skin inflammation characterized by neutrophil infiltration, epithelial disruption and keratinocyte cell death. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional protein widely known for its implication in the glycolytic pathway and other non-canonical function, such as cell death. Here, we report using a combination of pharmacological and genetic strategies that inhibition of nuclear translocation of Gapdh resulted in decreased skin neutrophil infiltration, speed and displacement length, and in reduced number of keratinocyte hyperproliferation and cell death. In addition, neutrophil number correlated with skin inflammation and expression in neutrophils of wild-type Gapdh, but not a nuclear translocation defective mutant, exacerbated skin inflammation. Similarly, inhibition of nuclear translocation of GAPDH reduced the expression of pathology-associated markers in human organotypic 3D skin models of chronic inflammation. Finally, single cell RNA-Seq analysis of Spint1a-deficient larvae revealed that inhibition of nuclear translocation of Gapdh reduced the expression of a set of genes that were induced in neutrophils and keratinocytes of inflamed skin larvae as well as neutrophil-specific *il4* and *ptpn6*. Strikingly, Il4 deficiency alleviated skin inflammation of Spint1a-deficient larvae, suggesting that Il4 production by neutrophils in response to the nuclear translocation of Gapdh contributed to skin inflammation. Overall, our data supports that a non-canonical function of GAPDH is an important mediator of acute and chronic skin inflammation by modulating both keratinocyte biology and neutrophil infiltration to skin lesions. This mechanism provides new insights into the contribution of neutrophils to acute and chronic skin inflammation, and can have a significant translational impact to develop new therapeutic approaches to treat skin inflammatory disorders.

LIST OF ABBREVIATIONS

- AB - Zebrafish line AB
- ACE2 - Angiotensin-Converting Enzyme 2
- AF - Atrial fibrillation
- AMPK - AMP-dependent Kinase
- AP-1 - Activator Protein-1
- APE1 - AP endonuclease 1
- ARDS - Acute Respiratory Distress Syndrome
- ATP - Adenosine triphosphate
- BCA - Bicinchoninic acid
- BFP - Blue fluorescent protein
- BLT-1 - LTB4 Receptor
- BSA - Bovine serum albumin
- C3a - Complement Component 3a
- C5a - Complement Component 5a
- Cas-9 - CRISPR-associated protein 9
- Cas13d - CRISPR-associated protein 13d
- CCAR2 - Cell cycle activator and apoptosis regulator 2
- CCL - CC-Chemokine Ligand
- CD41 - Cluster of differentiation 41
- CDC - Centers for Disease Control and Prevention
- CHT – Caudal Hematopoietic Tissue
- CMP - Common Myeloid Progenitor
- CNS - Central nervous system

- COVID-19 - Coronavirus Disease 2019
- CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats
- crRNA - CRISPR RNA
- CT - Computed Tomography
- CXCL - C-X-C Motif Chemokine
- CXCR - CXC Chemokine Receptor
- DAMPs - Damage-Associated Molecular Patterns
- DCs - Dendritic Cells
- DETCs - Dendritic Epidermal T Cells
- DMF - Dimethyl fumarate
- DMSO – Dimethyl Sulfoxide
- DNA - Deoxyribonucleic acid
- dsRED - Red fluorescent protein
- ECM - Extracellular Matrix
- EdU - 5-ethynyl-2'-deoxyuridine
- eGFP - Enhanced Green Fluorescent Protein
- FLG gene - Filaggrin gene
- fMLP - fMet-Leu-Phe
- FOV - Field of view
- FPR - fMLP Receptor
- GAIT - IFN-γ-activated inhibitor of translation
- Gal4.VP16 - Gal4 variant with VP16 activation domain
- GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
- GAPDHS - GAPDH, Spermatogenic
- GATA1a - GATA binding protein 1a

- GCL - Glutamate-Cysteine Ligase
- G-CSF - Granulocyte Colony-Stimulating Factor
- GEO - Gene Expression Omnibus
- GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
- GMP - Granulocyte-Macrophage Progenitor
- GSTM1 - Glutathione S-Transferase Mu 1
- Hai1a - Hepatocyte growth factor activator inhibitor 1a
- HIF-1α - Hypoxia-inducible factor 1-alpha
- HK1 - Hexokinase 1
- HMGB1 - High Mobility Group
- HOSCN - Hypothiocyanous acid
- hpf - Hours post-fertilization
- HSCs - Hematopoietic Stem Cells
- ICU - Intensive Care Unit
- IDT - Integrated DNA Technologies
- IFN - Interferon
- IFNG - IFN gamma
- IL - Interleukin
- ILCs - Innate Lymphoid Cells
- iNOS - Inducible nitric oxide synthase
- IRF3 - Interferon Regulatory Factor 3
- kDa - Kilodalton
- LC - Langerhans Cells
- LC3 - Microtubule-associated protein 1A/1B-light chain 3
- LCN2 - Lipocalin 2

- LDH - Lactate dehydrogenase
- LTB4 - Leukotriene B4
- LTB4R – LTB4 Receptor
- lyz - Lysozyme
- M1 macrophages - Classically Activated Macrophages
- M2 macrophages - Alternatively Activated Macrophages
- MAPKs - Mitogen-activated protein kinases
- M-CSF - Macrophage CSF
- mDCs - Myeloid Dendritic Cells
- MHC I - Major Histocompatibility Complex Class I
- MIP1 - Macrophage inflammatory protein 1
- MITO-Dendra2 - Mitochondria-targeted photoconvertible fluorescent protein
- MMF - Monomethyl fumarate
- MMPs - Matrix Metalloproteinases
- MOs - Morpholinos
- mpeg1 - Macrophage-expressed gene 1
- MPO - Myeloperoxidase (mammals)
- mpx – Myeloperoxidase (zebrafish)
- mRNA - Messenger Ribonucleic Acid
- MSR - Macrophage scavenger receptor
- mTOR - Mammalian target of rapamycin
- NAD^+ - Nicotinamide adenine dinucleotide (oxidized form)
- NADPH - Nicotinamide adenine dinucleotide phosphate
- NAMPT - Nicotinamide phosphoribosyltransferase
- NE - Neutrophil Elastase

- NES - Nuclear export signal
- NETosis - Neutrophil Extracellular Trap Formation
- NETs - Neutrophil Extracellular Traps
- NFkB-RE - Nuclear factor kappa-light-chain-enhancer of activated B cells response element
- nfsB - Nitroreductase nfsB
- NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
- NHS - National Health Service
- NLR - Neutrophil-to-Leukocyte Ratio
- NLRs - Nod-Like Receptors
- NLS - Nuclear localization sequence
- NO - Nitric oxide
- NOX - NADPH Oxidase
- O-GlcNAc - O-linked N-acetyl glucosamine
- ONOO- - Peroxynitrite
- OVOL1 - Epidermal Transcription Factor
- P2X7R - P2X7 Receptor
- PAMPs - Pathogen-Associated Molecular Patterns
- PARP1 - Poly(ADP-Ribose) Polymerase 1
- PBS - Phosphate-buffered saline
- PBST - PBS with Tween 20
- PCR - Polymerase Chain Reaction
- pDCs - Plasmacytoid Dendritic Cells
- PDGF - Platelet-Derived Growth Factor
- PFA - Paraformaldehyde

- PI3K-Akt - Phosphoinositide 3-kinase-Akt
- PKB - Protein kinase B
- PMA - Phorbol Myristate Acetate
- PPARγ - Proliferator-Activated Receptor-γ
- PPE - Personal Protective Equipment
- PRMT - Protein Arginine Methyltransferase
- PRRs - Pattern Recognition Receptors
- PTM - Posttranslational modification
- RLRs - Rig-like Receptors
- RNA - Ribonucleic acid
- RNS - Reactive nitrogen species
- ROI - Region of interest
- ROS - Reactive Oxygen Species
- S100A8/A9 - S100 calcium-binding protein A8/A9
- SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2
- SGLT2i - Sodium glucose co-transporter 2 inhibitor
- SIAH1 - Seven in Absentia Homolog 1
- SIN-1 - linsidomine chlorhydrate
- SNO-GAPDH - S-nitrosylated GAPDH
- SOD1 - Superoxide Dismutase 1
- Spint1a - Serine peptidase inhibitor, Kunitz type 1a
- Tg - Transgenic
- TGFβ - Transforming Growth Factor Beta
- TH - T Helper
- TL - Zebrafish line TL

- TLR - Toll-Like Receptor
- TNF - Tumor Necrosis Factor
- tracrRNA - Trans-activating CRISPR RNA
- UAS - Upstream Activation Sequence
- UVB - Ultraviolet B Light
- VDAC1 - Voltage-dependent anion channel 1
- VEGF - Vascular Endothelial Growth Factor
- VHD - Valvular heart disease
- WHO - World Health Organization
- WIK - Zebrafish line WIK
- XO/XDH - Xanthine Oxidase/Dehydrogenase
- ZIRC - Zebrafish International Resource Center

INTRODUCTION

Immunity and the Immune system

The immune system is a mechanism that all living organisms, from sea sponges to humans, present and allow them to distinguish between their internal environment and external factors. This ability defends the organisms against external agents that can compromise their survival (McComb, Thiriot, Akache, Krishnan, & Stark, 2019). The ability of the organisms to defend against diseases is the immunity.

Immunity can be separated in two categories depending on the type of response carried out: humoral and cell-mediated. Humoral immunity is a response consisted of the immune cells-mediated release of molecules such as antibodies, cytokines or complement proteins in order to neutralize or remove external material called antigens. Cell-mediated immunity is driven by immune system cells which response directly to an antigen (Cavaillon, 2011; McComb et al., 2019).

Moreover, immune system has two main branches: the innate and the adaptive immune system (Dempsey, Vaidya, & Cheng, 2003). The majority of the organisms are able to survive with only innate immune system, but higher vertebrates developed another defense system called adaptive immune system (Dempsey et al., 2003; Mantovani, Cassatella, Costantini, & Jaillon, 2011). A strong cross-play between innate and adaptive immune response is crucial for an accurate regulation (Dempsey et al., 2003).

Innate immune system

Innate immunity provides, together with the natural barriers of the organism, the first line of defense against external pathogens and microorganisms, providing crucial mechanisms that guarantee a fast sensing and removing of the external agents. The immune cells that participate in the innate immunity are dendritic cells (DCs), mast cells, eosinophils, basophils, natural killer (NK) cells and the most important, macrophages and neutrophils (**Figure 1**). These cells identify pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) by the use of membrane receptors called pattern recognition receptors (PRRs), in order to trigger membrane trafficking that will lead to the initiation of the immune response (Chaplin, 2010; Taguchi & Mukai, 2019). The humoral innate immune response is based on the secretion of components like naturally occurring antibodies, pentraxins, antimicrobial peptides,

complement proteins, lipopolysaccharide (LPS) binding protein or mannose binding lectin (Chaplin, 2010; Shishido, Varahan, Yuan, Li, & Fleming, 2012; Turvey & Broide, 2010). Humoral immunity has a close relationship to autoimmune disorders. Some pathogens are able to use humoral components to increase pathology (Shishido et al., 2012).

Although it has been commonly known that the immunological memory is exclusive from the adaptive immune response, recent reports have shown that innate immune cells show adaptive features. This innate immune property has been called “trained immunity”, and refers to the functional reprogramming of innate immune cells which triggers an altered and more effective response, adjusted to an accurate context and time depending on the pathogen, in a second exposition to the same antigen (Naik et al., 2017; Netea et al., 2020).

Adaptive immune response

Higher vertebrate organisms have evolved developing the adaptive immune response, that provides a broader variety of responses that allow the recognition of self- and nonself-antigens (Bonilla & Oettgen, 2010). Cell-mediated adaptive immune response is carried out by lymphocytes, which can be divided into T and B cells (**Figure 1**). These cells suffer the rearrangement of gene segments, generating new genes that codify specific antigen receptors, called T cell receptors (TCRs) or B cell receptors (BCRs). Lymphocytes, by a cross-play with innate immune cells like DCs or macrophages, involved in antigen presentation, recognize the antigens and activate the adaptive immune response, generating immunological memory. After the first recognition of a pathogen, long-lived memory lymphocytes are generated, staying in the tissues and being quickly activated in the subsequent encounters with the same antigen (Bonilla & Oettgen, 2010). The humoral adaptive immune response is based on the B cell-mediated release of antibodies, responsible of the detection of pathogens and its bound, neutralizing them or activating phagocytic or oxidative activity of the innate immune cells like macrophages (Bonilla & Oettgen, 2010; Turvey & Broide, 2010; Viana et al., 2021). Recently, an innate part of the adaptive immune system has been described. The presence of innate B cells, like B1 and marginal zone B cells, and innate lymphoid cells (ILCs), which carry out innate-like activities, has triggered the actual reevaluation of the innate and adaptive immune system dichotomy (Hillion et al., 2020).

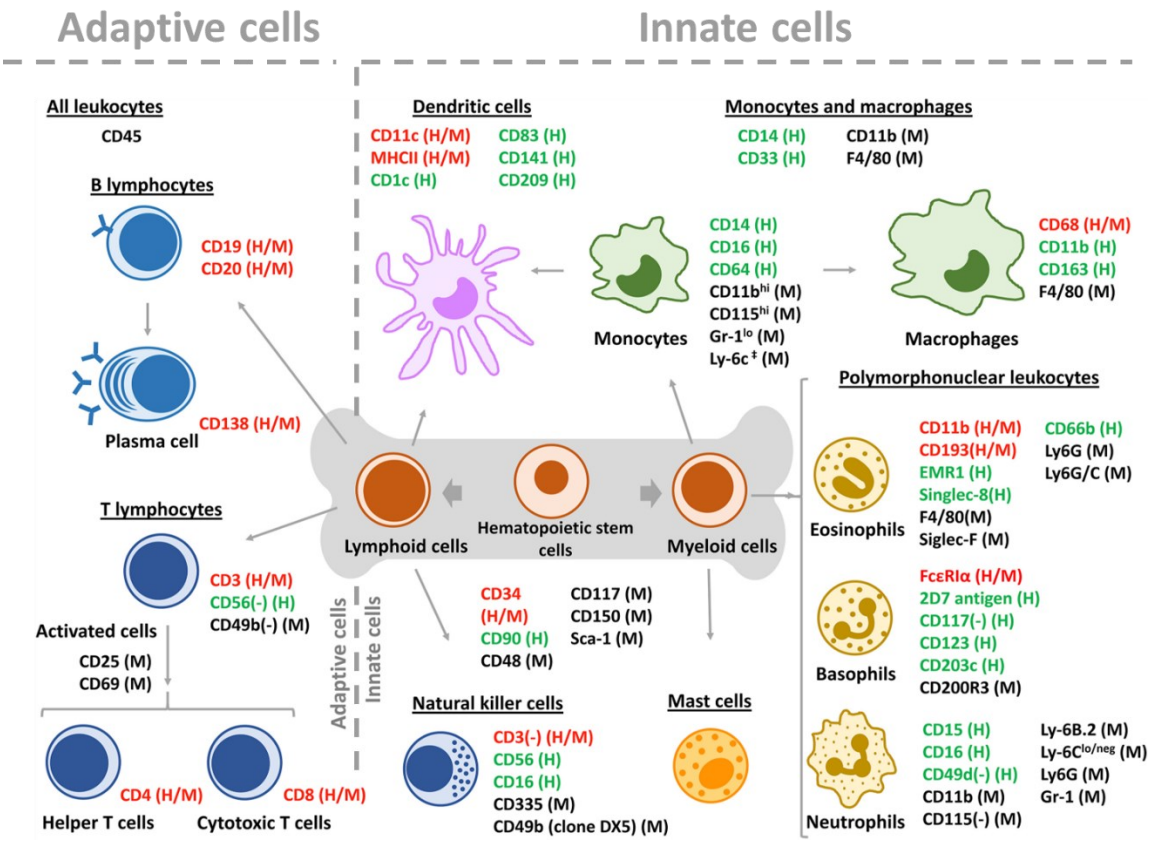

Figure 1. Innate and adaptive immune cells. Immune response can be divided in two categories: innate, involved in the first defense mechanism of the organism; and adaptive, a more complex response that provides long-lasting defense and memory against pathogens. On the one hand, the innate immune system includes cells like dendritic cells, monocytes, macrophages, mast cells, natural killer cells and the granulocytes: eosinophils, basophils and neutrophils. On the other hand, lymphocytes are the main cells in the adaptive immune system. B lymphocytes can be found as pre-B, B lymphocytes or plasma cell; and T lymphocytes as Helper, Cytotoxic or Regulatory T cells (adapted from Viana et al., 2021).

Inflammation

Inflammation is a response, immune or adaptive, which consists of a broad variety of physiological and pathological processes whose main goal is to recover tissue homeostasis. These processes are usually provoked by conditions which lead to a loss of structure, such as tissue injury, tissue stress or malfunction; or infections, that induce the recruitment of leukocytes and plasma proteins to the inflamed tissue (Medzhitov, 2008, 2021).

Recently, it has been described that transient or permanent loss of function of particular proteins caused by noxious substances can activate inflammatory response. Moreover, loss of regulation of homeostasis can activate inflammatory response in the absence of infection, tissue damage or exposure to noxious substances, usually as a response to factors such as cold, starvation or

predation. Loss of function and loss of homeostatic regulation are scenarios which need further investigation (Akdis, 2021; Medzhitov, 2021). There are five major signs (ancients called them cardinal signs) of inflammation: heat (*calor*), redness (*rubor*), swelling (*tumor*), pain (*dolor*), and loss of function (*functio laesa*) (Hurley, 1964; Punchard, Whelan, & Adcock, 2004).

Inflammation can be classified according to the time of activity into three categories: acute inflammation, that occurs during few days; subacute inflammation, that lasts between two and six weeks; and chronic inflammation, that is active during months or even years (Hannoodee & Nasuruddin, 2023; Pahwa, Goyal, & Jialal, 2023).

Several stimuli are able to activate inflammatory response. All of them can be divided in two main categories: exogenous and endogenous inducers. Among the exogenous causes, the microorganisms are the most studied. Microbial inducers present PAMPs, such as LPS, which are conserved structures present in microbes that can be recognized PRRs of the immune system cells. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and the mannose receptor are examples of PRRs expressed in immune system cells (Mogensen, 2009). The PAMP-PRR interaction induces the release of cytokines and chemokines that recruit other immune cells, triggering the initiation of the immune response. Apart from PAMPs, some microbes can release virulence factors that are also able to provoke the inflammatory response. There are also exogenous non-microbial inducers of inflammatory response, like allergens, toxins or other foreign bodies (**Figure 2**) (Hannoodee & Nasuruddin, 2023; Valles, Lorenzo, Bocanegra, & Valles, 2014; Varela, Mogildea, Moreno, & Lopes, 2018).

Endogenous factors, known DAMPs, can also induce inflammation. DAMPs are released as a result of different cellular processes like cell death, damage or stress and, in spite of their contribution to the host defense, they can also induce inflammation. These patterns are also recognized by PRRs and are reportedly involved in diseases such as Alzheimer's disease, Parkinson's disease or rheumatoid arthritis, among others (T. Gong, Liu, Jiang, & Zhou, 2020; Roh & Sohn, 2018; Valles et al., 2014).

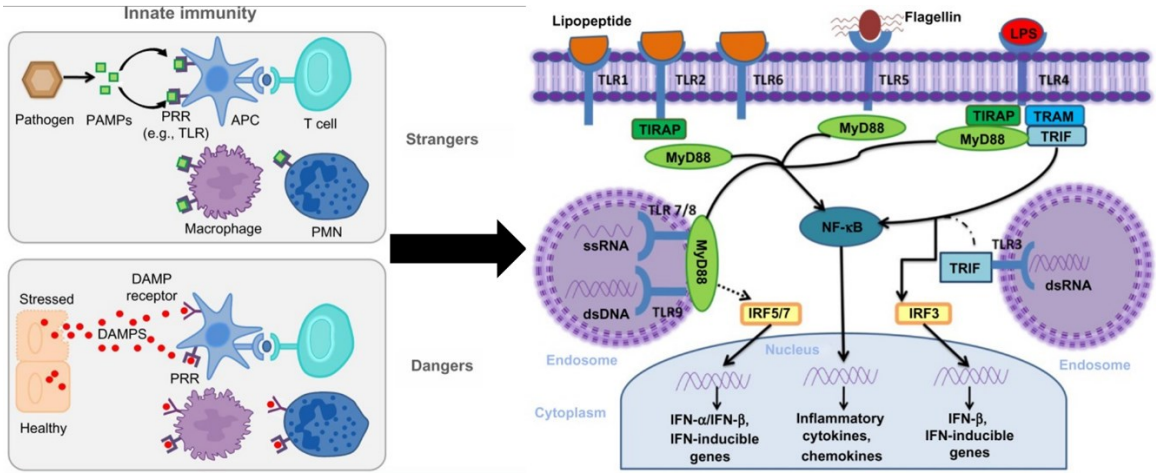

Figure 2. PAMPs and DAMPs recognition pathway. PAMPs and DAMPs are recognized by PRRs, triggering the initiation of the innate immune response in the different immune cells and the regulation of the subsequent adaptive immune response. One of the most important PAMPs are TLRs, which are able to recognize PAMPs like LPS, lipopeptide or flagellin, and DAMPs like ssRNA or dsRNA. Pattern recognition activates NF-κB pathway, via TIRAP-TRAM-TRIF-MyD88 axis, leading to the production of inflammatory cytokines which will recruit more immune system cells. LPS, lipopolysaccharide; TLR, toll-like receptors; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; MyD88, myeloid differentiation factor 88; IFN, interferon; ssRNA, single strand ribonucleic acid; dsDNA, double-strand deoxyribonucleic acid; dsRNA, double-strand ribonucleic acid; IRF, IFN regulatory factor; TRAM, TRIF-related adaptor molecule; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptors; TLR, toll-like receptor; DAMPS, danger-associated molecular patterns; APC, antigen-presenting cells; PMN, neutrophils. Adapted from Valles et al., 2014.

Depending on the stimuli, the inflammatory response tries to reestablish the tissue homeostasis in different ways: in an infection, the response provides the host a defense against the microorganism infecting the tissue (Cohen, 2002); in a tissue injury, the inflammatory response repairs the damaged tissue (Eming, Wynn, & Martin, 2017); and during a stress stimuli, like in post-traumatic stress disorder (PTSD), the inflammatory response allows the tissue adaptation to the stress, leading to the restoration of homeostatic state (Hori & Kim, 2019; Speer, Upton, Semple, & McKune, 2018). In order to report the transition between full inflammation response and homeostasis, researchers stablished a term called para-inflammation. Para-inflammatory response includes different inflammatory states that can fluctuate from being close to the homeostasis state to a full inflamed context (Medzhitov, 2008, 2010; Varela et al., 2018).

Unfortunately, under some contexts, inflammatory response can be deregulated, triggering negative effects in the organism than can result in numerous human disorders and pathologies (Marchi, Guilbaud, Tait, Yamazaki, & Galluzzi, 2023; Medzhitov, 2008). These disorders are different among them. On the one hand, inefficient inflammatory responses in an infection context result in sepsis, characterized by a damaging host response that trigger a tissue damage that can provoke eventually an organ failure (**Figure 3**) (Cohen, 2002; Gusev & Zhuravleva, 2022; van der Poll, van de Veerdonk, Scicluna, & Netea, 2017). Furthermore, disproportionate

autoimmune reactions are involved in the triggering and progression of diseases such as type 2 diabetes (Prasad, Chen, Toh, & Gascoigne, 2020). On the other hand, beyond infectious and autoimmune disorders, disproportionate inflammation can drive neurological diseases, as Parkinson disease (Tansey et al., 2022), digestive diseases like chronic inflammatory bowel disease (Roda et al., 2020), cardiovascular diseases such as thrombosis (Stark & Massberg, 2021), and renal diseases, among others (Basso, Andrade-Oliveira, & Camara, 2021; Marchi et al., 2023). Moreover, a defective inflammatory response or resolution could facilitate the progression and survival of malignant cells, promote metastasis, or even alter the response to chemotherapy or drug treatments (Coussens & Werb, 2002; Mantovani, Allavena, Sica, & Balkwill, 2008; Mantovani, Garlanda, & Allavena, 2010).

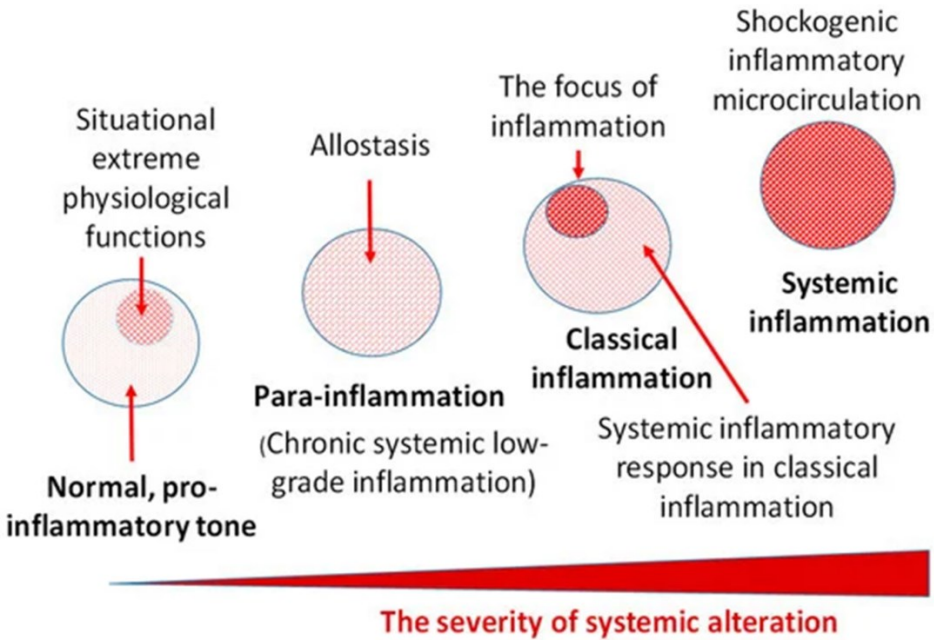

Figure 3. Severity of systemic alteration. During the inflammatory progression, in a first step, cells can suffer normal tissue stress conditions. The physiological tissue stress can trigger para-inflammation, a chronic and variable inflammatory state that can fluctuates with the progression of the inflammation. The tissue can also suffer the classical inflammation, characterized for being a local injury that can trigger a systemic inflammatory response. Last, in some specific conditions, a systemic inflammation can be triggered, leading to systemic shock and microvascular injuries. Modified from Gusev, 2022

Acute inflammation

In the early phase of acute inflammation, PAMPs or DAMPs of the affected tissue are recognized by the receptors present in the surfaces of resident tissue macrophages and dendritic cells. The stimulation of those receptors activates, via myeloid differentiation 88 (MyD88), the activation of the transcription factor Nuclear factor-κB (NF-κB), inducing the expression and the release of a large number of inflammatory cytokines like TNF-α, IL-1β, IL-6 or CXC motif chemokines such as CXC motif chemokine ligand 8 (CXCL8) (T. Liu, Zhang, Joo, & Sun, 2017) (**Figure 2**). Other group

of mediators activated in acute inflammation are the complement proteins, mainly C3a and C5a, that can activate neutrophils, monocytes and mast cells (Hannoodee & Nasuruddin, 2023).

Apart from NFκB, there are others transcriptional factors involved in inflammatory response initiation, such as interferon regulatory factor 3 (IRF3), which induces the expression of IFNs, IL-12 and IL-23 (Takeuchi, 2012); or the activator protein-1 (AP-1), which shares its targets with NF-κB (L. Chen et al., 2018).

At the same time, mast cells are activated by tissue damage, releasing histamine TNF-α, kinin and leukotrienes. Kinin can be transformed into bradykinin, a molecule that increases the permeability of the endothelium and the expression of adhesion markers which allow the leukocyte migration and extravasation (Bossi, Peerschke, Ghebrehiwet, & Tedesco, 2011; Theoharides et al., 2012).

Neutrophils are the next immune system cells to be recruited to the inflamed tissue. These cells release reactive oxygen species (ROS) and enzymes, removing dead cells and killing infectious microbes, and other chemokines that attract more neutrophils and monocytes to the tissue. In an early phase, the monocytes differentiate into M1 macrophages, releasing ROS and recruiting immune cells. In a later phase, they differentiate into M2 macrophages, releasing growth factors such as vascular endothelial growth factor (VEGF) or platelet-derived growth factor a (PDGFA), and antiinflammatory cytokines like IL-10 or transforming growth factor-β (TGFβ) to promote tissue repair (Oishi & Manabe, 2018; Varela et al., 2018). Eventually, macrophages release resolvins, protectins and maresins, lipid mediator which drive the resolution of the tissue injury, finalizing the inflammatory response (Watanabe, Alexander, Misharin, & Budinger, 2019).

Chronic inflammation

Chronic inflammatory diseases represent the main cause of death in the world. Worldwide, three of five people die due to chronic inflammatory diseases like stroke, chronic respiratory diseases, heart disorders, cancer, obesity and diabetes (Pahwa et al., 2023; Tsai et al., 2019). The effects and the extent of chronic inflammation depend on the cause of the injury and the ability of the body to repair the damage, being risk factors an old age, obesity, a diet rich in saturated fat, smoking, low sex hormones, stress and sleep disorders (Furman et al., 2019; Pahwa et al., 2023).

The chronic inflammation can be generated when the immune system is not able to eliminate the infectious organism or the foreign material that is triggering the acute inflammation; when the immune system cells are defective, generating autoimmune disorders such as rheumatoid arthritis, or autoinflammatory disorders such as Familial Mediterranean Fever; when there are

recurrent episodes of acute inflammation; or when the acute inflammatory response causes oxidative stress and mitochondrial dysfunction (**Figure 4**) (Baniyash, 2004; Pahwa et al., 2023).

About the pathophysiology of chronic inflammation, vasodilation, blood flow and capillary permeability allow leukocytes to be recruited. When neutrophils are not able to remove the inflammatory cause, they die and are replaced by lymphocytes, plasma cells and macrophages. Macrophages, unable to switch from a M1 to a M2 phenotype, overproduce ROS, proinflammatory cytokines, granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), involved in the recruitment of new neutrophils and the expansion of their lifespan, avoiding the resolution of the inflammatory response (M. Li, Hou, Zhong, Zhao, & Fu, 2021). Lymphocytes B and T produce cytokines to costimulate each other and to recruit new leukocytes, and also antibodies and immunocomplexes to fight the inflammation (Pahwa et al., 2023).

There is an important crosstalk between immune cells in chronic inflammation, with neutrophils secreting extracellular traps to stimulate macrophage cytokine production (Castanheira & Kubes, 2019), macrophage interacting with endothelial cells to trigger neutrophil recruitment (Ren et al., 2023), or macrophages and lymphocytes interacting through the antigen presentation process to stimulate each other and prolong or perpetuate immune response (Germolec, Frawley, & Evans, 2010; Germolec, Shipkowski, Frawley, & Evans, 2018).

Persistent chronic inflammation can result in the activation of local tissue fibroblast, which increase the production of extracellular matrix (ECM) components that accumulates in the tissue, forming a fibrotic tissue and fibrosis. Every organ is susceptible of suffering fibrosis, and when this fibrosis is extended, it could induce defects in the tissue morphology, organ dysfunction and, eventually, the organ failure (Henderson, Rieder, & Wynn, 2020).

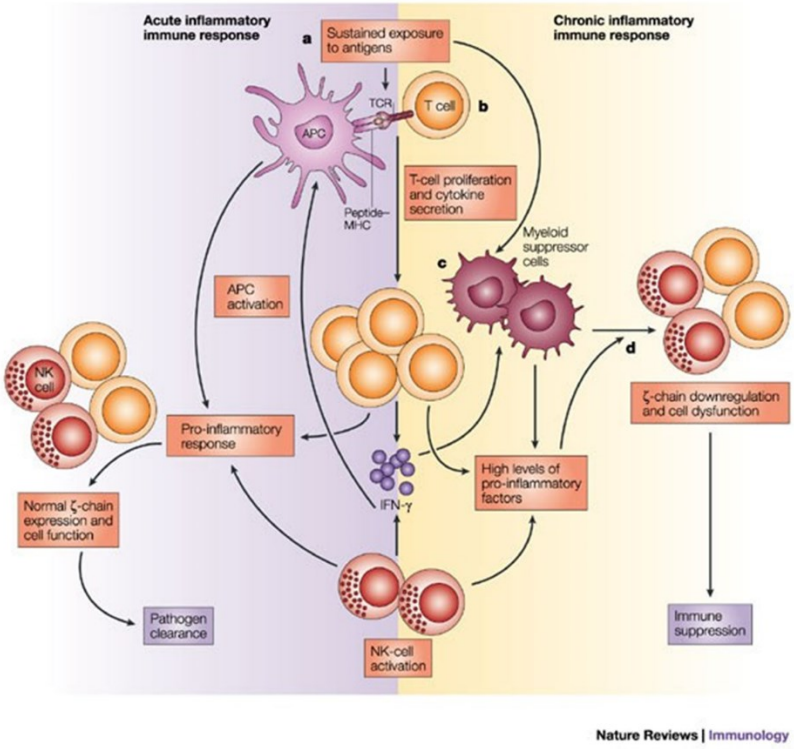

Figure 4. Acute and chronic inflammatory immune response. In acute inflammation, tissue resident cells like DCs or macrophages recognize the antigens and activate pro-inflammatory response. This cells also act as antigen presenter cells (APC), activating lymphocytes and NK cells to carry out the pathogen clearance. In addition, when the acute inflammatory response is not enough or is defective, it can trigger chronic inflammation, where leukocytes generate high levels of pro-inflammatory cytokines, immune cell dysfunction and immune suppression. NK, Natural killer; APC, antigen presenter Cell; IFN-γ, Interferon gamma; TCR; T Cell receptor; MHC, Major Histocompatibility Complex. Adapted from Baniyash, 2004.

The skin and its associated inflammatory disorders

The skin: a vital barrier for the organism

The skin constitutes one of the main barriers of the body against exogenous stresses and factors that can be harmful. This multi-layered structure is the largest organ in the organism, and is formed by a huge variety of cell populations organized in three different layers: epidermis, dermis and hypodermis.

Keratinocytes are the main epidermal population, although melanocytes and Merkel cells can also be found as important structural components of this layer. Keratinocytes express a huge amount of PRRs in their surface, including TLRs, Rig-like receptors (RLRs), NLRs and inflammasomes. The epidermis also present immune system cells such as Langerhans Cells (LC), a self-maintained subset of tissue-resident macrophages which migrates to lymph nodes and present antigens to T cells (Doebel, Voisin, & Nagao, 2017). Other adaptative immune system cells found in the epidermis are the resident dendritic epidermal T cells (DETCs), a subset of T Helper and T cytotoxic cells that stay in the epidermis after infections, and also ILCs, which have

a similar shape and cytokine release pattern to T cells but do not present antigen receptors (Clottu, Humbel, Fluder, Karampetsou, & Comte, 2021; Kobayashi, Naik, & Nagao, 2019; Ricardo-Gonzalez et al., 2018).

The dermis is a supportive layer of connective tissue that provides structure and stability to the skin. It is mainly formed by fibroblasts which, by secreting collagen fibers, proteoglycan and elastin, create an organized cell matrix where immune cells like macrophages, mast cells, dendritic cells, T cells, eosinophils and basophils are embedded, ready to carry out immune responses or wounds healing (**Figure 5**) (Kabashima, Honda, Ginhoux, & Egawa, 2019; Kobayashi et al., 2019; Pasparakis, Haase, & Nestle, 2014).

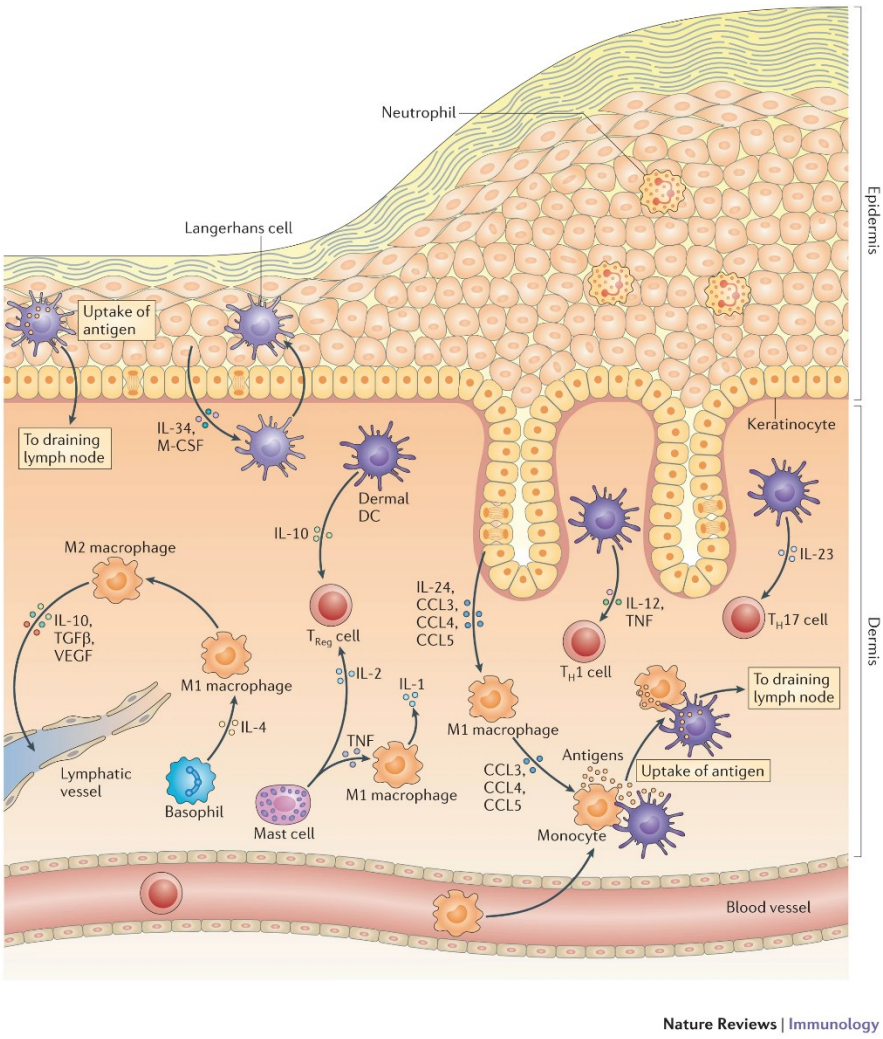

Figure 5. Skin layers and immune cell composition. In the skin, epidermis and dermis can be observed, each one with a different type of cells. The dermis is formed by keratinocytes, and some individual cells can be found infiltrated, like Langerhans cells, macrophages or neutrophils. The dermis contains a broad variety of cell types, including, apart from keratinocytes, fibroblasts, monocytes, macrophages, basophils, mast cells, memory and effector T cells, and DCs. IL, interleukin; M-CSF, Macrophage cell stimulating factor; TGFβ, Tumor Growth Factor β; VEGF, Vascular Endotelial Growth Factor. Adapted from Pasparakis et al., 2014.

Below the dermis, the hypodermis is a subcutaneous layer composed by connective and adipose tissue. Adipocytes play a diverse variety of roles, among them, they are able to regulate the skin temperature, to provide a connection between the skin and muscles, to store fat and energy, and to regulate hair growth and mediate antimicrobial immune response. Moreover, macrophages, T cells and B cells reside also in this layer (Guerrero-Juarez & Plikus, 2018; Kabashima et al., 2019; Kobayashi et al., 2019; L. J. Zhang et al., 2015).

The microbiota that covers the different surfaces of the skin has also a strong influence in the immune processes, affecting innate and adaptative responses (**Figure 6**) (Kong & Segre, 2012). The microbial composition of the skin microbiota fluctuates depending on the individual and his physiology, being *Propionibacterium, Staphylococcus* and *Corynebacterium* species the most abundant generally. Skin microbiota plays an important role in preparing the immune system cells presented in the skin. For example, *S. epidermidis* secreted products are able to recruit a population of cytotoxic T cells which express cytokines like IL-17A or IFN in absence of inflammation (Byrd, Belkaid, & Segre, 2018; Kabashima et al., 2019; Linehan et al., 2018). Common inflammatory diseases, like acne, eczema, atopic dermatitis, psoriasis or chronic wounds, are associated with processes of dysbiosis that occur in the skin microbiota (Byrd et al., 2018). Antibiotic treatments against dysbiosis could be enough to avoid it and to eliminate skin inflammation, like the use of creams with steroids and antibiotics in children with severe atopic dermatitis (Dallo, Patel, & Hebert, 2023).

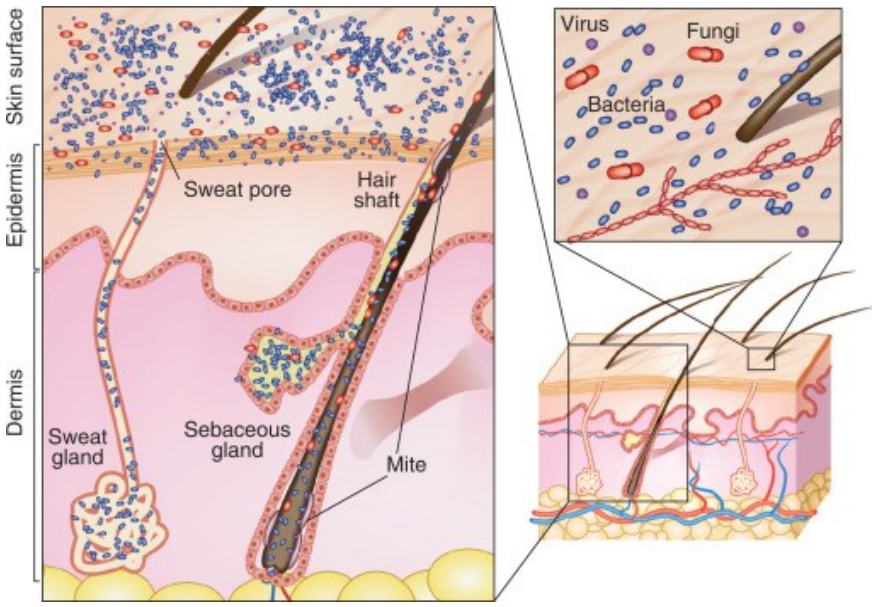

Figure 6. Microbiota presence in the different skin layers. The different microorganisms that form the skin microbiota are present covering the surface of the skin and in the sebaceus and sweat glands and also in the hair. (Kong & Segre, 2012)

Skin inflammatory disorders

Defective or disproportionate immune responses in the skin, due to a dysregulation in the immune cell function, lead to the development of inflammatory skin diseases. These diseases are generally multifactorial and the molecular mechanisms and causes remain unclear. Conventional treatments with corticosteroids have focused on alleviating the symptoms. Nowadays, new treatments focus in the specific cytokines produced in each disease or in the enzymes involved in the characteristic signaling pathways of the disorders (Song, Lee, & Kim, 2022).

In this work, we are going to focus in two of the main chronic skin inflammatory disorders worldwide: psoriasis and atopic dermatitis.

Psoriasis

Psoriasis is a chronic skin immune-mediated disease which affects a percentage of the global population between 2-3%. This incidence seems to be increasing over time and nowadays affects more than 125 million people worldwide. It is generally characterized by a dysregulation in the keratinocyte proliferation, followed by the infiltration of immune system cells which release inflammatory cytokines. The long-term activation of the inflammatory response causes a broad damage in the skin that could affect other tissues and organs (Greb et al., 2016; Lebwohl, 2003).

There are several manifestations of psoriasis (**Figure 7**) (Dhabale & Nagpure, 2022; Greb et al., 2016):

- The most common is plaque-type psoriasis, also known as psoriasis vulgaris. This kind is characterized by dry skin, itchiness and the appearance of silvery scales covering the skin. It is commonly manifested in joints, scalp and trunk.
- Psoriasis guttate is the second type of psoriasis with a higher incidence. It is often triggered by bacterial infection with *Streptococcus*. It is characterized by the appearance of small papules and can start in the childhood or adolescence.
- Inverse psoriasis is often manifested at the same time of other subtypes of psoriasis. In this case, the presence of scales is not necessary, being more represented by the appearance of erythematous plaques.
- Pustular psoriasis appears mainly in the hands and feet of adults and it’s characterized by erythema, sterile pustules and scales.
- The most severe kind of psoriasis, that can be life-threatening, is erythrodermic psoriasis. It causes a widespread erythema in the body that triggers pain and itching. It is generally an unstable manifestation of psoriasis vulgaris.

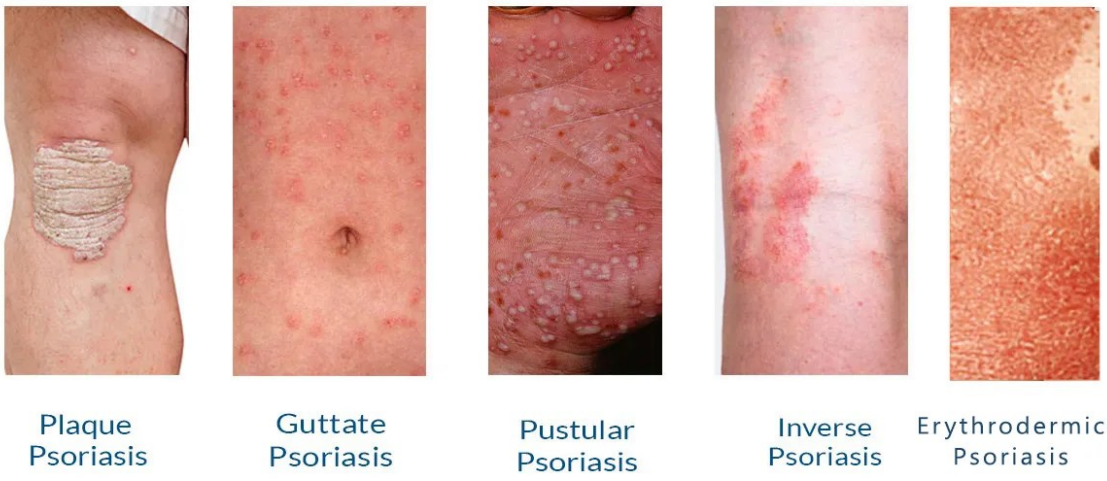

Figure 7. Types of psoriasis. Some of the most common clinical manifestations of psoriasis are plaque psoriasis (most common), guttate, pustular, inverse or erythrodermic psoriasis. Plaque psoriasis is characterized by dry skin and silver scales, guttate psoriasis by small papules, pustular psoriasis by erythema, sterile pustules and scales, inverse psoriasis by erythematous plaques, and erythrodermic by widespread erythema. Source: WebMD (https://www.webmd.com/).

One of the factors that determine the manifestation of the disease is the genetic component. Linkage and genetic association studies have identified more than 70 genes and 17 susceptibility loci associated with psoriasis. Examples of these genes are *CARD14*, *IL23R*, human leukocyte antigens (*HLAs*) or *PTPN22*, among others (Greb et al., 2016; Tsoi et al., 2015; Tsoi et al., 2012).

Factors like trauma, bacterial infection, or medication can trigger psoriasis. This situations trigger the release of self-nucleotides that can form a complex with the cathelicidin antimicrobial peptide produced and released by the epidermal keratinocytes. Dendritic cells' TLRs recognize the complex and trigger antigen presentation and later activation of the CD8+ T cells that are initially in the epidermis and dermis, that after being activated, interact with keratinocytes promoting proliferation. Dendritic cells also release cytokines like TNF-α, IL-12 and IL-23, triggering, together with IL-1β, a T_H17 response, characterized by the release of IL-17 by the T cells promoting the recruitment of immune cells, like macrophages, mast cells or neutrophils. The expression of IL-22 by other T cells provokes a T_H22 response, that triggers hyperplasia, acanthosis and paraketosis in the skin; and the activation of the NF-κB pathway in keratinocytes, that eventually triggers the release of new cytokines, like TNF-α or CCL20 (Greb et al., 2016).

Psoriasis has been associated to hypertension, type 2 diabetes, cardiovascular disease, Chron's disease, cancer, psoriatic arthritis (psA) and metabolic syndrome (MetS) (Fatas-Lalana et al., 2022; Hao et al., 2021; Yamazaki, 2021). T_H17 cells and the release of IL-17 is one of the main characteristics of the mechanism of psoriasis, and also, one of the reported links between psoriasis and the comorbidities associated to the disease, such as MetS. Nevertheless, the

correlation between psoriasis and the comorbidities remains poorly known and controversial (Greb et al., 2016; Yamazaki, 2021).

There is no specific cure for the psoriasis disease, but several treatments have been developed. For patients with mild psoriasis, the main therapy consists of a topical treatment with corticosteroids, calcineurin inhibitors, keratolytics or vitamin D analogues, together with phototherapy. In the situation of a severe psoriasis, treatments that inhibit TNF-α, p40IL-12/23, IL-17 or phosphodiesterase 4 are used (Armstrong & Read, 2020; W. Y. Chen et al., 2022). (Table)

Atopic dermatitis

Atopic dermatitis is a chronic skin inflammatory disorder manifested as a recurrent, localized eczema, that carries dry skin, skin lesions and lichenification, and is usually developed in childhood. This disease is one of the most prevalent inflammatory skin disorders, with an increasing incidence between 15 and 20% in children and around 10% in adults worldwide (Kolb & Ferrer-Bruker, 2023; Stander, 2021).

The manifestations of the disease are variable. The skin lesions are characterized by redness (erythroderma), papules, edema and the appearance of scales, and might localize widespread on the body, but more frequently in trunk and ventral wrists (**Figure 8**) (Kolb & Ferrer-Bruker, 2023; Leung & Bieber, 2003; Stander, 2021).

Atopic dermatitis has a strong genetic component, with 34 susceptibility loci identified, with genes involved in immune response, type-2 differentiation, innate immunity, epidermal differentiation and T cell activation. Among them, one of the main genes involved in atopic dermatitis development is filaggrin, encoded by the *FLG* gene (Kolb & Ferrer-Bruker, 2023; Langan, Irvine, & Weidinger, 2020; Martin et al., 2020; Paternoster et al., 2015).

Although the factor that trigger atopic dermatitis remains unclear, it is well-known that several factors, genetic and environmental, interacts between them provoking the skin inflammation. Among them, microbial dysbiosis, epithelial disruption and immune deregulation are one of the principal factors. A reduction in the microbial diversity, with a great abundance of *Staphylococcus aureus*, and a deficiency in the epidermal barrier, increase the susceptibility to skin inflammation, activating a subtype of LCs present in the skin which express IgE in their membranes. The recognition of allergens or antigens in the skin by this subpopulation of LCs triggers the release of cytokines which activates ILC2 cells in the dermis, producing IL-13 and IL-5, recruiting and activating eosinophils, neutrophils and T_H2 cells, and generating an acute lesion. T_H2 cells release IL-4, IL-13 and IL-31, activating B cells in the skin. The T_H2 response dysregulation

induces itch, the lichenification of the skin, and the establishment of a chronic skin lesion (Kolb & Ferrer-Bruker, 2023; Langan et al., 2020; Stander, 2021).

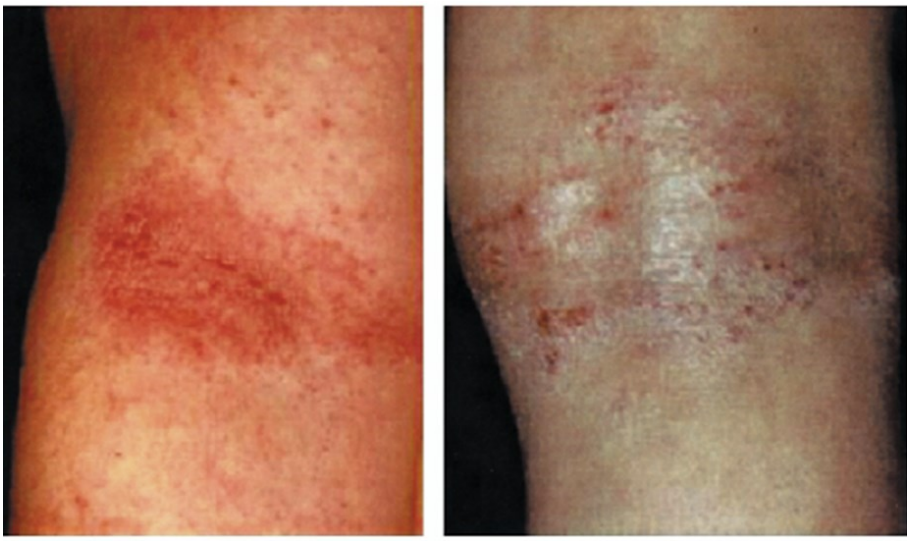

Figure 8. Atopic dermatitis. Atopic dermatitis lesions are characterized by redness, papules, edema and the appearance of scales. Under some circumstances, they can also suffer lichenification, with acanthosis in the epidermis, spongiosis and paraketosis Modified from Leung & Bieber, 2003.

A situation of persistent atopic dermatitis could carry another transient complication, like asthma, rhinitis, rhinoconjunctivitis, or food allergy, in relation with the skin barrier disruption and a defective T_H2 response in the skin. Atopic dermatitis is associated to other comorbidities such as cardiovascular and metabolic diseases, anxiety and depression (Berce, Marhold, & Delopst, 2022; Silverberg, 2019).

As in psoriasis, the treatment for atopic dermatitis depends on the clinical stage of the disease. When moderate, phototherapy with ultraviolet A or B is usually applied, meanwhile when severe, therapies with glucocorticoids, cyclosporine or methotrexate are frequently used. More recently, new therapies against IL-4, IL-13, IL-22, Janus kinases (JAKs) and phosphodiesterase 4 have been developed (W. Y. Chen et al., 2022; Stander, 2021).

Psoriasis and atopic dermatitis overlap.

Psoriasis and atopic dermatitis are different chronic skin inflammatory disorders (**Figure 9**). On the one hand, psoriasis is characterized by a T_H17 response, in which IL-17 is one of the main cytokines involved. On the other hand, atopic dermatitis triggers a T_H2 and T_H22 response, with cytokines like IL-4, IL-13 or IL-22 involved in the development of the disease. Despite this, recently, an overlap of atopic dermatitis and psoriasis have been observed in 1.3% of the patients diagnosed with either psoriasis or atopic dermatitis, although due to the difficult diagnosed of the diseases, a higher percentage of prevalence of both diseases is expected (Barry et al., 2021).

Some similarities have been recently described between psoriasis and atopic dermatitis. In some populations like Asia, atopic dermatitis shows a strong T_H2 and T_H22 activation, but also T_H17, and presents epidermal paraketosis, acanthosis and a neutrophil infiltration more similar to the observed in psoriasis (Guttman-Yassky & Krueger, 2017; Noda et al., 2015). Also, psoriasis and atopic dermatitis manifest some similarities. Recent reports show that they present an increase in the B cell-derived IgE levels in serum, *parthanatos* cell death of keratinocytes, secretion of adipokines in the adipose tissue, or a similar predisposition to obesity, among other common patterns (Ali, Ulrik, Agner, & Thomsen, 2018; Arroyo et al., 2023; L. Luo et al., 2022; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021; Nowowiejska, Baran, & Flisiak, 2023). Although the overlap between both diseases is rare, the similarities are driving to the development of some therapies that could treat both diseases, such as Poly ADP-ribose Polymerase 1 (PARP1) inhibitors, or JAK1 inhibitors (Arroyo et al., 2023; Gargiulo et al., 2023).

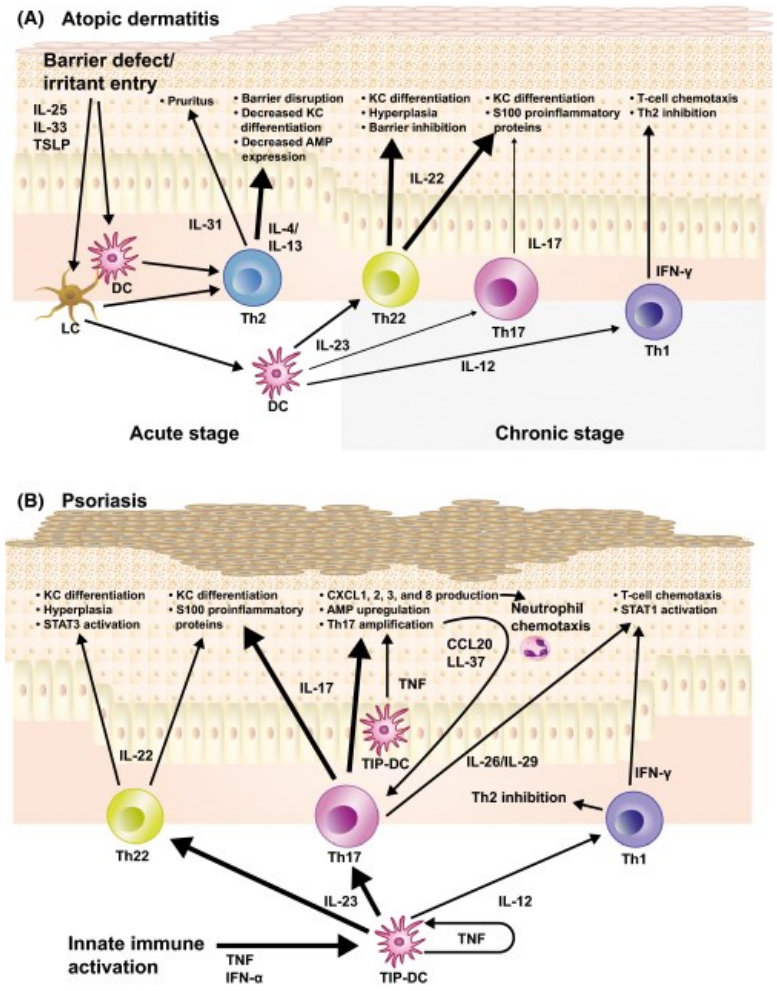

Figure 9. Immune system mechanisms in chronic skin disorders psoriasis and atopic dermatitis. (A) Atopic dermatitis is characterized by the presence of DCs and LCs that trigger T_H2 response, leading to changes in keratinocyte differentiation, hyperplasia or barrier inhibition. In the chronic stage, T_H1, T_H22 and T_H17 responses are combined. **(B)** Psoriasis is characterized by a IL23-induced T_H17 response that works with a T_H22 and T_H1 response during the progression of the disease. AMP, antimicrobial peptide; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; DC, dendritic cell; IFN, interferon; IL, interleukin; KC, keratinocyte; LC, Langerhans cell; LL-37, cathelicidin peptide; STAT, signal transducer and activator of transcription; Th, T helper; TIP-DC, tumor necrosis factor and inducible nitric oxide synthase producing dendritic cell; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin (Guttman-Yassky & Krueger, 2017).

Oxidative stress in chronic skin inflammation

Oxidative stress was introduced as an overproduction of oxidative molecules that antioxidants cannot remove, resulting in a damage of biological structures (Sies & Cadenas, 1985). The term oxidative stress involves molecules derived from O_2, called ROS, and the products derived from N_2, called reactive nitrogen species (RNS). They can be also recognized together as reactive oxygen and nitrogen species (RONS).

ROS are generated in metabolic pathways such as mitochondrial respiratory chain, the xanthine oxidase (XO or XDH) activity, or the NADPH oxidases (NOX1, NOX2, NOX4, NOX5, DUOX1 and

DUOX2) activity (R. P. Brandes, Weissmann, & Schroder, 2014; Forman & Zhang, 2021). Some of the members of the NOX family have constitutive activation (NOX4), others depend on cytosolic activators (NOX1, NOX2 and NOX3), and the rest are calcium-dependent (DUOX1, DUOX2 and NOX5) (**Figure 10**) (R. P. Brandes et al., 2014). These reactions involve the reduction of O_2, which generates molecules such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), ozone or hydroxyl radicals (·OH). RNS are produced mainly by the activity of Nitric Oxide Synthases (NOSs), which produce Nitric Oxide ($^{\cdot}NO$) by the oxidation of L-arginine. The rest of RNS derived from this $^{\cdot}NO$, which, after reacting with the ROS, generates peroxynitrite ($ONOO^-$), a very strong antioxidant (Al-Shehri, 2021; Calabrese et al., 2007; Forman & Zhang, 2021).

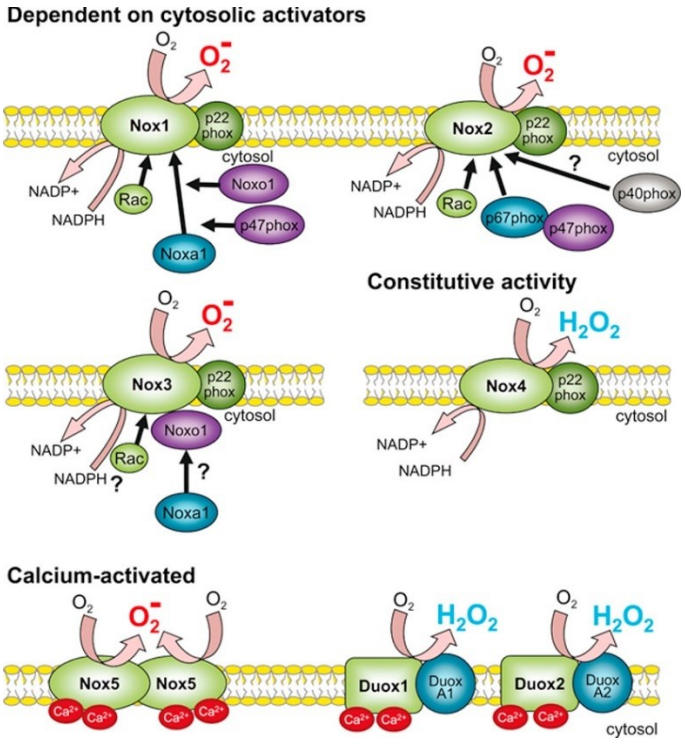

Figure 10. NADPH oxidase protein family. NOXs are one of the most important ROS producers in the cell. There have been reported seven members in the family, NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. NOX1-3 are dependent on cytosolic activators: NOX1 depends on NOXA1, whose activity is regulated by NOXO1 and p47PHOX; NOX2 is regulated by RAC, p67PHOX, p47PHOX and probably p40PHOX; and NOX3 is regulated by NOXO1 and RAC, and probably NOXA1. These enzymes produce anion superoxide after their reaction. NOX4 has constitutive activity and generates hydrogen peroxide. NOX5, DUOX1 and DUOX2 are calcium dependent. NOX5 generates anion superoxide and DUOX1-2 depends on DUOXA1 and generate hydrogen peroxide. NADPH, Nicotine Adenine Dinucleotide Phosphate (reduced); NOX, NADPH oxidase; NOXA1, NOX Activator 1; Rac, Rac GTPase; Ca^{2+}, cation calcium; DUOX, Dual Oxidase; O_2^-, anion superoxide; H_2O_2, hydrogen peroxide (R. P. Brandes et al., 2014).

RONS have been reported as important factors in the development of different diseases, such as pulmonary fibrosis, Alzheimer disease, hypertension, type 2 diabetes mellitus, or cancer (Forman & Zhang, 2021).

Antioxidant enzymes provide a defense mechanism for the organism, repairing oxidative damage and removing the oxidative stress. Activation of transcription factors like AP-1, proliferator-activated receptor-γ (PPARγ) or NF-κB, induces the expression of antioxidant genes such as catalase, Superoxyde Dismutase 1 (*SOD1*), glutamate-cysteine ligase (*GCL*) or glutation S-transferase Mu 1 (*GSTM1*) (Al-Shehri, 2021; Forman & Zhang, 2021).

In psoriasis and atopic dermatitis, the abundance of immune system cells, overall neutrophils and lymphocytes, results in the production of huge amounts of RONS. In psoriasis, macrophages are the main RONS producers, recruiting neutrophils and lymphocytes that increase the oxidative stress. The levels of antioxidants like catalase and vitamin E in the blood is reduced in psoriasis patients (Pujari, Ireddy, Itagi, & Kumar, 2014). Some therapies with antioxidants have shown promising results in the treatment of psoriasis disorders, such as curcumin, sylimarin or antioxidant senolytics (Guarneri, Bertino, Pioggia, Casciaro, & Gangemi, 2021; Hernandez-Silva et al., 2022; Nakai & Tsuruta, 2021). Atopic dermatitis patients show a low concentration of antioxidant vitamins like vitamin E and vitamin C in blood and in skin lesions. N-Acetyl cysteine treatment results were promising as an atopic dermatitis therapy, suppressing T_H2 response (Nakai & Tsuruta, 2021; Nakai et al., 2015).

Neutrophils

Neutrophils are the more abundant leukocytes present in the human blood, representing approximately a 70% of the circulating leukocytes. This type of polymorphonuclear leukocyte represents one of the main defense mechanisms against inflammation, being the first leukocytes recruited when an inflammatory stimulus appears (Kolaczkowska & Kubes, 2013; Nathan, 2006; Phillipson & Kubes, 2011). Although neutrophils are considered short-life cells, with a half-life of around 5 days in peripheral blood, their life span remains controversial because of the criticism that the methodological approach received, stable isotope labeling studies, due to its instability and the different conditions the experiments were carried out (Hidalgo & Casanova-Acebes, 2021; Hidalgo, Chilvers, Summers, & Koenderman, 2019; Lahoz-Beneytez et al., 2016; Tofts, Chevassut, Cutajar, Dowell, & Peters, 2011). The neutrophil life span is extended in inflammatory or infectious conditions (Ley et al., 2018).

As a response to a defective immune response, the long-term accumulation of neutrophils in tissues or organs can trigger an ample damage in the compartment (Brewer, 2023; Kolaczkowska & Kubes, 2013; Phillipson & Kubes, 2011). Moreover, neutrophils accumulation in solid tumors is correlated with bad prognosis (Gungabeesoon et al., 2023).

Neutrophil differentiation

Hematopoietic stem cells (HSCs) are a stem cell population with self-renewal and the ability to generate erythroid, myeloid and lymphoid cells. Several processes control the HSCs differentiation. In a strong inflammatory context, for example, a process called cell danger mobilization occurs, leaded by the effect of the G-CSF-mediated activation of the NLRP3 inflammasome, increasing the demand for myeloid cells (neutrophils and monocytes) to fight against the inflammatory stimulus (Lenkiewicz et al., 2019; Ratajczak, Kim, et al., 2010; Ratajczak, Lee, et al., 2010; Thapa et al., 2021; Yanez et al., 2017).

A HSCs committed to a myeloid differentiation, located in the bone marrow and extramedullary tissues, is differentiated to the common myeloid progenitor (CMP). In the first steps, a CMP is differentiated to a granulocyte macrophage progenitor (GMP). This cell has the capacity of generating any myeloid cell, depending on their molecular pattern of expression: monocytes, eosinophils, mast cells, basophils or neutrophils (Hidalgo et al., 2019; Ramirez & Mendoza, 2018; Yanez et al., 2017).

After committing to neutrophil differentiation, a GMP generates a promyelocyte, and this one a myelocyte. At this point, in bone marrow, a pool of myelocytes called “lazy pool” stay resting without differentiating, and the other pool of cells, called postmitotic neutrophil pool, differentiates into banded neutrophils, and later into mature neutrophils (Hidalgo et al., 2019). At this time, in bone marrow, there are different neutrophil subsets specialized in specific functions like expansion, trafficking and effector, ready to take part and act in stress conditions (Evrard et al., 2018; Huerga Encabo et al., 2023).

Recently, single cell data identified an early specific population of GMPs, that they called proNeu, that showed an early commitment to neutrophils, challenging the GMP definition (**Figure 11**) (Calzetti et al., 2022; I. Kwok et al., 2020; Ng, Liu, Kwok, & Ginhoux, 2023; Ng, Ostuni, & Hidalgo, 2019). This proNeu population shows a huge plasticity, being able to generate neutrophil-like monocytes under inflammatory conditions (Ikeda et al., 2023).

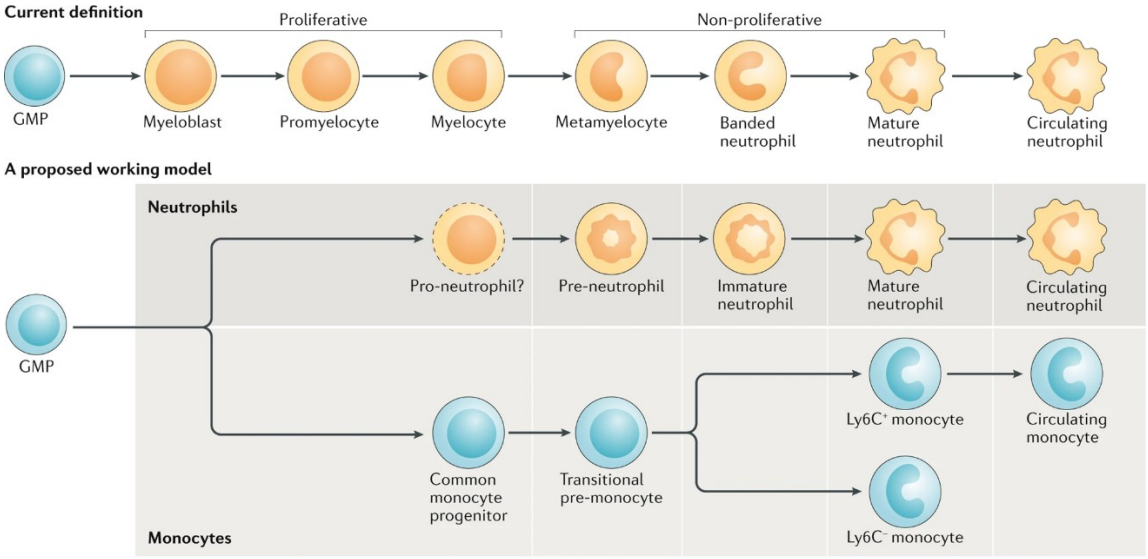

Figure 11. The neutrophil differentiation pathway. Granulocyte-monocyte progenitors (GMPs) are the precursor cells that will be differentiated into neutrophils. In the current definition of the neutrophil differentiation, a proliferative GMP differentiates into myeloblast, promyelocyte and myelocyte, which maintain their proliferative state; next, myelocyte is differentiated into metamyelocyte, which is not able to proliferate, banded neutrophil and mature neutrophil. Finally, the mature neutrophils will go out from the bone marrow as a circulating neutrophil. Nowadays, the new proposed model early GMPs can be compromised to neutrophils, called pro-neutrophil, a "common neutrophil precursor". These pro-neutrophils will generate mature neutrophils, or even neutrophil-like monocytes (not shown in the scheme). This proposed model challenges the classical granulopoiesis mechanism. Adapted from Ng et al., 2019.

Although it is commonly known that neutrophils finish their differentiation into mature neutrophils in the bone marrow, and then they are mobilized to the circulation, showing a single continuum across biological compartments (Grieshaber-Bouyer et al., 2021; Hidalgo et al., 2019), it remains controversial because of the reported presence of immature neutrophils in peripheral blood or infiltrated in organs like spleen (Hidalgo et al., 2019; Jhunjhunwala et al., 2016; Jordan et al., 2017; Massberg et al., 2007). Recent reports have described how severe inflammatory conditions lead to an abnormal release of immature neutrophils into the circulation, called "emergency granulopoiesis", that occurs in diseases like cancer or inflammation (**Figure 12**) (Manz & Boettcher, 2014; Scapini, Marini, Tecchio, & Cassatella, 2016). Although more research is needed, these circulating immature neutrophils has shown an increased bactericidal function, limited mobility, low phagocytic capacity and a mitotic capacity (Deniset, Surewaard, Lee, & Kubes, 2017; Grieshaber-Bouyer & Nigrovic, 2019; Uhl et al., 2016; L. Wang, Luqmani, & Udalova, 2022). These immature or aberrant neutrophils generated by emergency granulopoiesis have recently shown a role in immune suppression during sepsis and aggravation of stroke pathology in aged patients (Gullotta et al., 2023; A. J. Kwok et al., 2023).

Finally, aged neutrophils can suffer a recruitment back to bone marrow in a circadian manner, in a process known as "homing". Once in the bone marrow again, these aged neutrophils are

removed by macrophages in order to maintain homeostasis (Nauseef & Borregaard, 2014; Qu, Jin, Zhang, & Ng, 2023) .

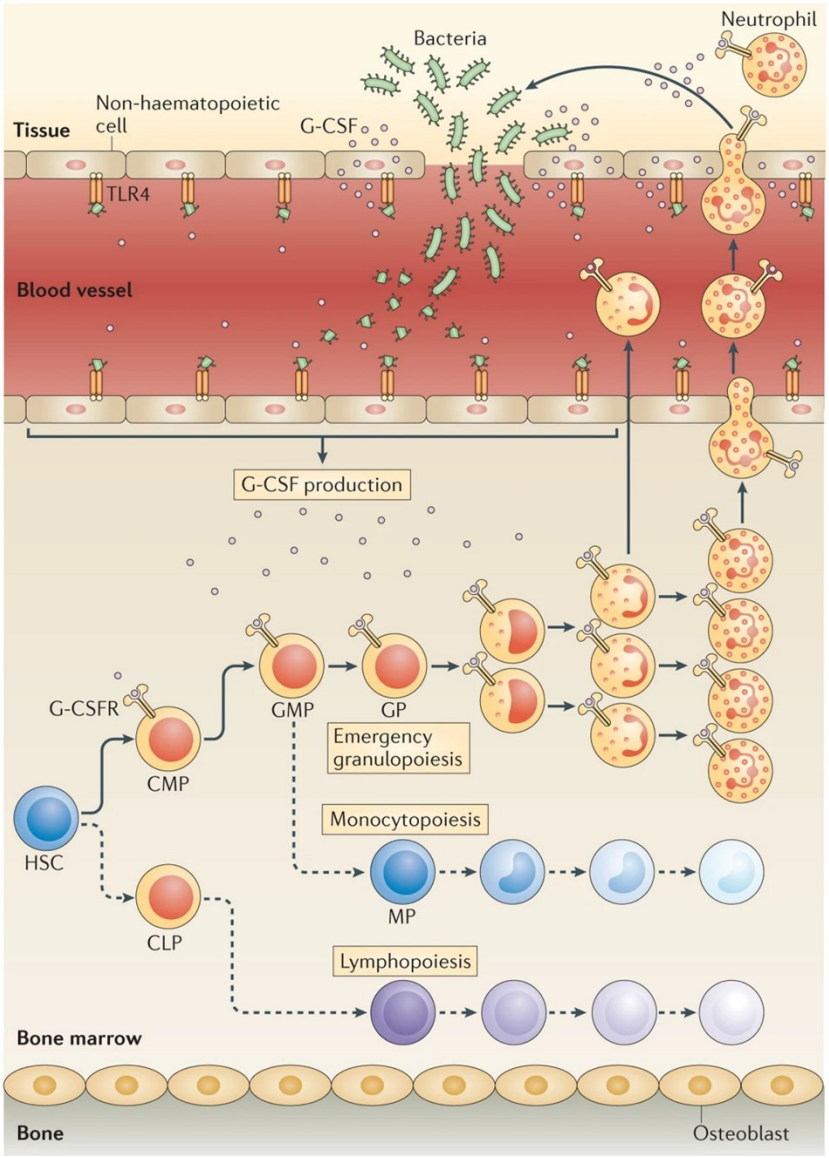

Figure 12. Emergency granulopoiesis. During an infection, PRRs, like TLR4, recognize PAMPs initiating the emergency state. TLR4 signal transduction carries out the secretion of G-CSF, stimulating neutrophil mobilization from the bone marrow to the periphery. Moreover, G-CSF increases neutrophil generation, decreasing lymphopoiesis and monocytopoiesis. In a severe inflammatory context, the neutrophils do not have enough time to be generated, what carries out not only the mobilization of mature neutrophils, but also the mobilization of immature neutrophils to the circulation and the inflamed tissue. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte–macrophage progenitor; GP, granulocyte progenitor; HSC, haematopoietic stem cell; MP, monocyte progenitor. Adapted from Manz & Boettcher, 2014.

Neutrophil migration.

During an inflammatory context, affected tissues secreted a huge amount of diverse chemoattractive molecules that immediately activate neutrophil mobilization and recruitment to the affected tissues (de Oliveira, Rosowski, & Huttenlocher, 2016).

Neutrophil recruitment from the vasculature to the tissue.

Neutrophil migration is a multistep process. Inflammatory cytokines released by activated-tissue resident macrophages induces the fast expression of P and E-selectins, which interacts with P-selectin glycoprotein ligand 1 (PSGL1) and leukocyte selectins (L-selectins) triggering the neutrophil capture. As the interaction between selectins is weak, the neutrophil continues rolling on the surface of the endothelial cells. This interaction activates the neutrophil expression of lymphocyte function-associated antigen 1 (LFA1). LFA1 recognizes intercellular adhesion molecule 1 (ICAM1) and ICAM2 specifically expressed in the endothelium. The strong resulting interaction induces the neutrophil full arrest and adhesion to the blood vessel surface (Filippi, 2019; Kolaczkowska & Kubes, 2013; Ley, Laudanna, Cybulsky, & Nourshargh, 2007). Neutrophil dynamics in the blood are importantly influenced by circadian rhythms, being a major factor involved in cytoskeleton rearrangement and resulting in a defective capacity of neutrophils to roll during daytime in mice (Adrover et al., 2019; Hidalgo et al., 2019; Ovadia, Ozcan, & Hidalgo, 2023).

After cell adhesion, neutrophils generate pseudopods and continue crawling towards endothelial junctions. This crawling is produced due to the interaction between ICAM1 and neutrophils MAC1. When neutrophils find an ample endothelial junction, a region with low matrix protein expression, or a gap between pericytes, they do diapedesis, the process by which neutrophils transmigrate to reach the inflamed tissues or organs. Neutrophils have two ways of crossing endothelial cells: paracellular, transmigrating between endothelial cells, or transcellular, transmigrating through endothelial cells (**Figure 13**) (Filippi, 2019; Kolaczkowska & Kubes, 2013; Ley et al., 2007).

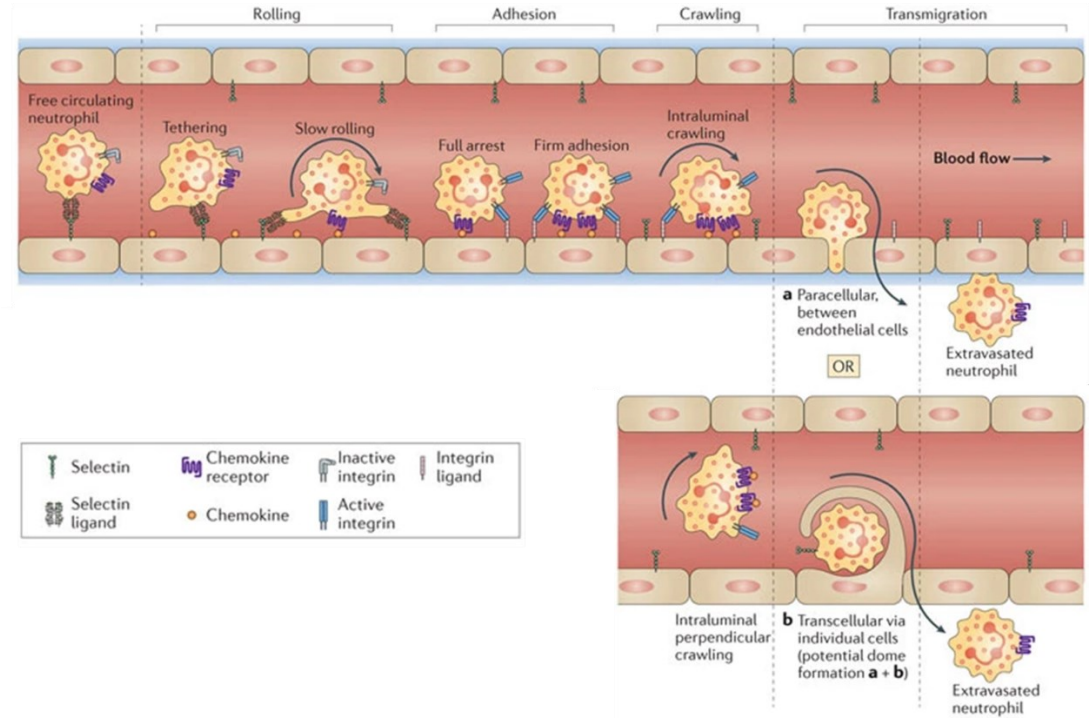

Figure 13. Steps in the neutrophil recruitment to the tissue. Free circulating neutrophils, after activation, start expressing membrane proteins that interact with the membrane of endothelial cells in the rolling phase. After this, neutrophils suffer a full arrest in the adhesion phase, which finish with the crawling. Finally, neutrophils suffer transmigration, that can be paracellular (a), migrating between endothelial cells, or transcellular (b), through endothelial cells. Adapted from Kolaczkowska & Kubes, 2013.

Early neutrophil recruitment

During the neutrophil early recruitment phase, neutrophils recognize DAMPs like ATP or uric acid by PRRs recognition, such as TLRs and NLRs (de Oliveira et al., 2014; de Oliveira et al., 2016). ROS, overall DUOX1-produced H_2O_2, are also directly sensed by neutrophils, activating SRC family kinase (SFK) LYN and leading to the neutrophil recruitment (**Figure 14**) (de Oliveira et al., 2016; S. K. Yoo, Starnes, Deng, & Huttenlocher, 2011). One of the chemoattractants activated by ATP and H_2O_2 is the CXC-chemokine ligand 8 (CXCL8) (de Oliveira, Boudinot, Calado, & Mulero, 2015; de Oliveira et al., 2014). Several CXCL chemokines are involved in neutrophil migration, being recognized by the G protein-coupled receptors (GPCRs) called CXC receptor 1 and 2 (CXCR1 and CXCR2). CXCL8 gradients formed in the inflamed tissue are able to recruit distant neutrophils (Sarris et al., 2012).

Among the neutrophil chemoattractants, we also find lipid mediators like arachidonic acid-derived leukotriene B4 (LTB4), recognized by LTB4R, and also N-formyl peptides, such as fMet-Leu-Phe (fMLP), that derived from infectious bacteria or damaged tissue and activates neutrophils through interacting with fMLP receptor 1 (FPR1), FPR2 and FPR3 (de Oliveira et al., 2016).

Recent studies have reported tissue resident mast cells as critical initiating neutrophil infiltration in the inflamed tissues via TNF-α secretion (Dudeck et al., 2021).

Neutrophils response amplification: swarming.

After the early recruitment, activated neutrophils and tissue macrophages increase the release of proinflammatory cytokines like IL-1β or CXCL8, and other chemoattractants such as LTB4. Moreover, some of the neutrophils recruited suffer a stress-induced necrosis, releasing new DAMPs detected by tissue cells' PRRs that release more chemoattractants. This process amplifies the neutrophil response, increasing the neutrophil recruitment and carrying out a largely unknown well-conserved situation called swarming (**Figure 14**) (de Oliveira et al., 2016; Isles et al., 2021; Kienle & Lammermann, 2016; Mihlan, Glaser, Epple, & Lammermann, 2022; B. L. Rocha-Gregg & A. Huttenlocher, 2021).

Swarming consists on an organized attack of the neutrophils to fight against inflammatory stimulus that an individual neutrophil cannot afford, such as microbial clusters or tissue macrolesions (Golenkina et al., 2021; Hopke et al., 2020; Uderhardt, Martins, Tsang, Lammermann, & Germain, 2019). However, swarming is an aggressive neutrophil function that can be dangerous for the host, provoking an immune overactivation, functional tissue damage and even organ dysfunction and needs to be strictly regulated (M. Brandes, Klauschen, Kuchen, & Germain, 2013; Uderhardt et al., 2019).

In a first step, this swarming is controlled by tissue resident macrophages, which in the presence of a microlesion, sense the released DAMPs and trigger a "cloaking" mechanism surrounding the injury and preventing neutrophil swarming (Uderhardt et al., 2019). In response to macrolesions or microbial infections, macrophages cannot cloak the tissue injury and swarming is triggered, being connexins and "calcium alarm" signals in response to necrotic tissue crucial regulators of the process (Poplimont et al., 2020). In order to finish it, and as a self-regulatory mechanism of swarming, excess of LTB4 or CXCL8 and CXCL2 triggers a desensitization of GPCRs receptors to these signals, leading to the stop of the neutrophil recruitment (Kienle et al., 2021). Although neutrophil swarming is a really unknown process and further research has to be done to understand the mechanisms, new techniques and procedures have been developed to characterize and analyze these complex behavior patterns in neutrophils (Crainiciuc et al., 2022; Georgantzoglou, Matthews, & Sarris, 2021).

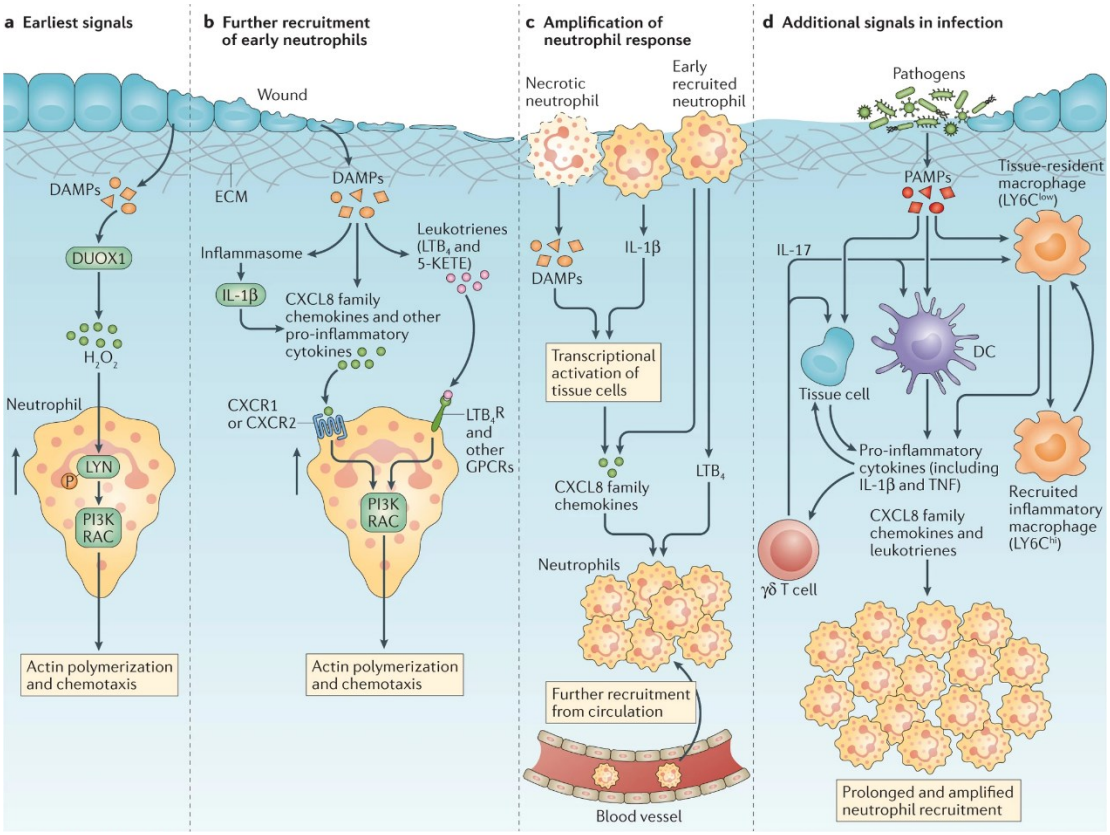

Figure 14. Phases of neutrophil recruitment to a damaged site. (a) DAMPs are released in the injury site by the affected cells, triggering the production and release of reactive oxygen species like H_2O_2, leading to the early recruitment of neutrophils through LYN kinase-PI3K-RAC axis, controlling actin polymerization and chemotaxis. **(b)** DAMPs can also induce the release of leukotrienes like LTB4 and 5-KETE, and the inflammasome activation, leading to the release of CXCL8, increasing neutrophil recruitment through interaction with the neutrophil receptors CXCR1 and CXCR2. **(c)** After activation of early recruited neutrophils, there is an increase in the secretion of CXCL8 and LTB4, amplifying the response and triggering neutrophil swarming. **(d)** During an infection, the process is similar. In this case, instead of DAMPs, PAMPs are detected and more signals like IL-17 are released, involving more immune cells, like tissue macrophages (resident and recruited), dendritic cells or T cells. 5-KETE, 5-oxo-eicosatetraenoic acid; CXCR, CXC-chemokine receptor; DUOX1, dual oxidase 1; ECM, extracellular matrix; GPCR, G protein-coupled receptor; IL, interleukin; LTB4R, LTB4 receptor; PI3K, phosphoinositide 3-kinase; TNF, tumour necrosis factor. Modified from de Oliveira et al., 2016.

Neutrophil reverse migration

In an acute inflammatory context, a correct clearance of the neutrophils after their function is critical to avoid host tissue damage and the appearance of a chronic inflammatory disease. At the inflammatory site, neutrophils suffered, depending on the conditions, different types of cell death like apoptosis, necroptosis or pyroptosis, and also lytic NETosis. After the cell death, the Kupfer cells and dendritic cells present in the tissues are the responsible of the removal of the neutrophils by phagocytosis (de Oliveira et al., 2016; Kolaczkowska & Kubes, 2013; Lawrence, Corriden, & Nizet, 2020). However, neutrophils do not need to die after their function, they can migrate to other tissues in a process called reverse migration (**Figure 15**) (de Oliveira et al., 2016; Ji & Fan, 2021; Lucas, Hoodless, & Rossi, 2014).

Although the mechanisms that leads the neutrophils to die or to reverse migrate remains poorly known, CXCR1 and CXCR2 are considered important molecules in the decision. CXCR1 has a really well-known role in neutrophil recruitment by interacting with CXCL8, but in the wound, the receptor is quickly internalized, meanwhile CXCR2 persists on the plasma membrane, triggering reverse migration and supporting the resolution of neutrophil clusters in the wound (Coombs et al., 2019; Ji & Fan, 2021; B. Rocha-Gregg & A. Huttenlocher, 2021).

To explain the different steps in neutrophil reverse migration, the authors described them as reverse abluminal crawling (rAC), reverse interstitial migration (rIM), reverse luminal crawiling (rLC), and reverse transendothelial migration (rTEM) (de Oliveira et al., 2016; Ji & Fan, 2021; Nourshargh, Renshaw, & Imhof, 2016).

Although neutrophil reverse migration allows the neutrophil resolution in the acute inflammatory tissue, it can be also a negative mechanism for the host. Neutrophils are activated and recruited to the inflammatory sites, and if they suffer reverse migration without being able to carry out their effector function, they suffer reverse transendothelial migration and move to other non-inflamed tissues or organs, contributing to their damage and disseminating the Inflammation in a systemic manner (Barkaway et al., 2021).

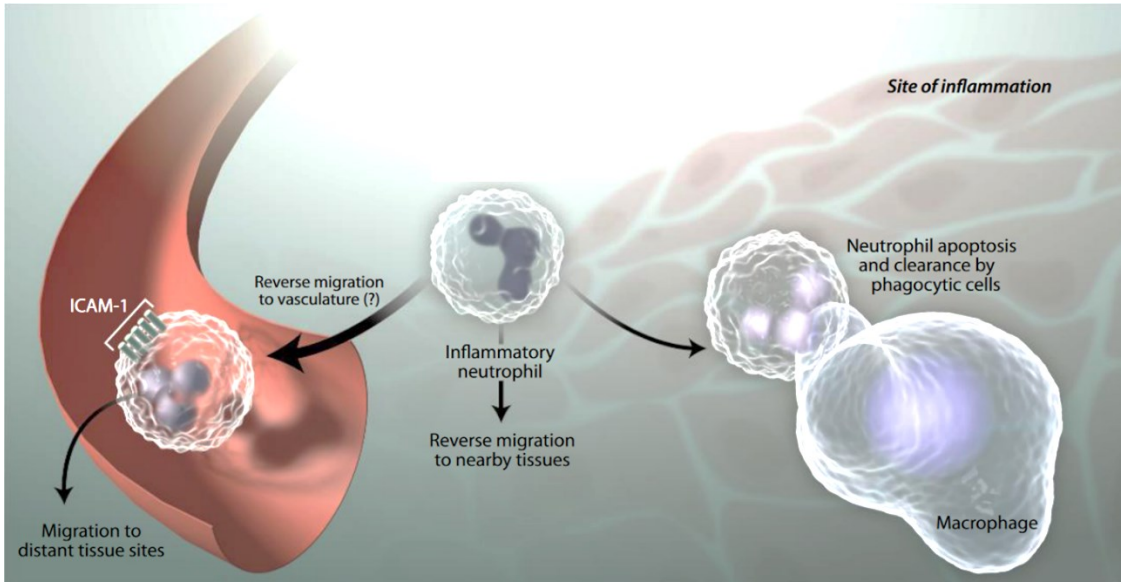

Figure 15. Inflammatory neutrophil fate after migration to the injury site. After migrating to the inflamed tissue, neutrophils can suffer cell death and being phagocyte by macrophages, or reverse migration to vasculature. Reverse migration also allows neutrophils to migrate to distant tissue sites, where they can carry out their functions. Adapted from Lucas et al., 2014.

Neutrophil effector functions

Despite being known in the past as cells with specific roles, neutrophils comprehend a cell type with a huge cellular heterogeneity and plasticity. Although neutrophil heterogeneity and

function have been widely studied in diseases, recent studies reported the presence of populations of tissue resident neutrophils infiltrated in the tissues in homeostasis conditions, acting as sentinels recognizing quickly injuries or microbial infections and triggering the recruitment of more immune cells (Ng et al., 2019; Silvestre-Roig, Fridlender, Glogauer, & Scapini, 2019; Yipp et al., 2017). In disease conditions, mature and immature neutrophils migrate to tissues where the tissue-derived signals determine their activation, differentiation and function are provided (**Figure 16**) (Ballesteros et al., 2020; Natoli & Ostuni, 2019; Rosales, 2018). Moreover, the crosstalk between neutrophils and other cell populations can reprogram neutrophil function in the inflamed tissue (Z. Gong et al., 2023).

Several neutrophil effector functions have been described in a disease context.

Respiratory burst.

In an infection context, immunocomplexes, bacterial fMLP, or the long-term exposure to inflammatory stimulus like G-CSF or TNF, lead to activate the assembly of the enzyme NOX2. This enzyme produces reactive oxygen species like anion superoxide ($O_2^{\cdot-}$) and H_2O_2, releasing them into the extracellular space or into phagosomes. Moreover, NOX2 activation leads to the release of myeloperoxidase (MPO), an enzyme included in the primary granules of the neutrophils. MPO uses the NOX2-derived H_2O_2 to produce hypochlorous acid, the most potent antimicrobial oxidant of the neutrophils, killing the infectious agent (L. Wang et al., 2022; Winterbourn, Kettle, & Hampton, 2016).

Phagocytosis.

Neutrophils are the most potent phagocyte cell. Phagocytosis consists of the quick uptake of infectious microbes by immune system cells in vesicles called phagosomes. Once inside the neutrophil, primary and secondary granules containing antimicrobial enzymes fuse with the phagosome, releasing all their content. Again, the respiratory burst generated by the NOX2 and MPO present in the neutrophil granules is essential killing the microbe (Burn, Foti, Marsman, Patel, & Zychlinsky, 2021; Liew & Kubes, 2019). Furthermore, in cancer conditions, neutrophils have been reported to phagocyte or trogocyte cancer cells. Trogocytosis consists of a leukocyte-mediated induction of cancer cell death by “biting” their plasma membrane (Reed, Reichelt, & Wetzel, 2021; Ustyanovska Avtenyuk, Visser, Bremer, & Wiersma, 2020).

Degranulation.

Neutrophil degranulation plays an important role in removing microbial pathogens. Neutrophils contain four different types of granules: primary (azurophil), which consist on MPO, defensins and proteases directly related to kill the pathogen; secondary (specific), which contain the iron

and copper-sequester lactoferrin; tertiary, which consist on gelatinase; and secretory granules containing albumin and cytokines (Liew & Kubes, 2019). In response to a pathogen, neutrophils suffer degranulation or exocytosis, releasing these granules to the extracellular space eliminating the pathogen. The order of granule mobilization is: secretory > tertiary > secondary > primary, being these last granules the main responsible of killing the microbe (Liew & Kubes, 2019; Mollinedo, 2019).

Neutrophil extracellular traps (NETs) formation and NETosis.

After sensing the presence of bacteria in the blood or in the tissues, apart from degranulating and phagocyting, neutrophils have developed a mechanism by which they release their DNA in a structure similar to a web containing histones, MPO, elastase and cathepsin G embedded in the net (Castanheira & Kubes, 2019). Initially, these structures were considered as a mechanism to trap and kill bacteria, and they were called neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Two different ways of NETosis have been described: lytic NET release, in which after the NETosis the neutrophil dies in a gasdermin D-dependent manner; and nonlytic NET release, in which the DNA nets are expelled via vesicles and the neutrophil does not die (Brinkmann et al., 2004; Castanheira & Kubes, 2019; Liew & Kubes, 2019; Thiam, Wong, Wagner, & Waterman, 2020). Platelets play an important role activating neutrophils and triggering NETosis via TLR4 and PSGL1, meanwhile DNA traps and histones released by neutrophils activate platelets, suggestion NETs as a coagulant factor (Palacios-Acedo et al., 2019).

NETosis and neutrophil degranulation are aggressive responses of neutrophils against pathogens. In situations where these processes are deregulated or are unable to eliminate the pathogen, the host cells and tissues can be widely damaged, triggering chronic inflammatory disorders and even predisposing the affected skin to tumorigenesis (Hoste et al., 2019).

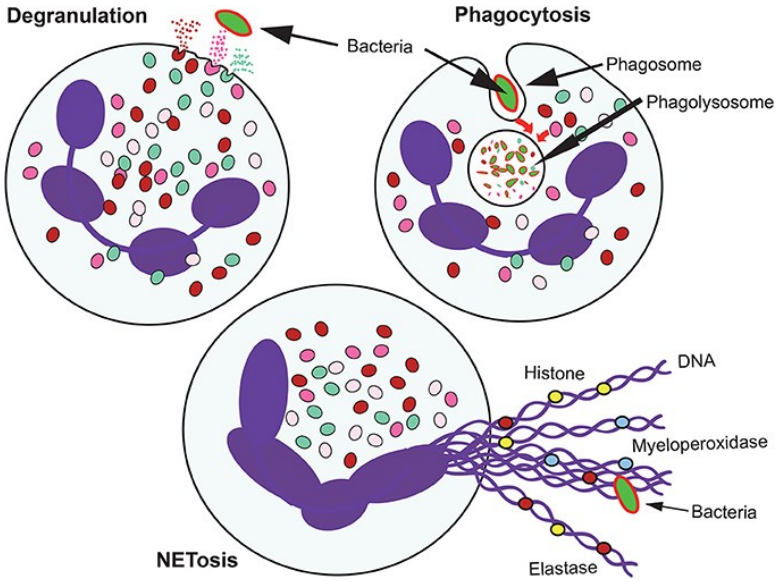

Figure 16. Neutrophil effector functions. After the recognition of the external agent, neutrophils carry out different effector functions to remove them. Neutrophils can carry out degranulation, releasing granules and vesicle to remove the pathogens; phagocytosis, internalizing the antigen in phagosomes and forming phagolysosome in order to remove the pathogen; or NETosis, releasing their DNA with a net-like structure together with histones and proteins like myeloperoxidase or elastase. Modified from Rosales, 2018.

Tissue repair.

Although the roles and functions of neutrophils in acute and chronic inflammation have been widely studied, some studies recently described a new role of neutrophils in tissue repair. After removing the infectious stimuli, neutrophils participate in remodeling the ECM by the secretion of Matrix Metalloproteinases (MMPs) such as MMP9. Furthermore, this MMP9, together with pyruvate kinase M2 (PKM2), is also able to promote the removal of dead and injured vessels and the following angiogenesis and lymphangiogenesis, healing wounds and injuries (Liew & Kubes, 2019; Massena et al., 2015; Shim, Deniset, & Kubes, 2022; Tan et al., 2013).

Neutrophils in skin inflammation

In a skin inflammatory context, neutrophil recruitment plays an important role in repairing the tissue and recovering homeostasis. Neutrophils act against microbial dissemination and secrete new factors to trigger tissue remodeling. The dark side of the process is that neutrophils require a straight regulation, otherwise their aggressive function could aggravate tissue damage and trigger chronic skin disorders (Phillipson & Kubes, 2019; Shim et al., 2022).

Neutrophils in skin wound

After the appearance of a wound in the skin, because of an injury or exposition to UVB, the wound healing process is activated, consisting of four phases: hemostasis, inflammation, proliferation and remodeling. In a first moment, in the hemostasis phase, platelets accumulation and vasoconstriction are enough to control the wound bleeding in case of a mild injury. In more

severe wounds, this response is not enough and the tissue response is the acute inflammation. DAMPs, calcium (Ca^{2+}) waves, and chemokines like CXCL8 or CXCL2 are secreted by death or activated keratinocytes, involved also in the production of PDGF and VEGF (Gurtner, Werner, Barrandon, & Longaker, 2008; Ohnstedt, Lofton Tomenius, Vagesjo, & Phillipson, 2019; Phillipson & Kubes, 2019; Poplimont et al., 2020). The secretion of these factors leads to the recruitment of immune cells, being the neutrophils the first leukocyte in being recruited to the skin (de Oliveira et al., 2016; Ellis, Lin, & Tartar, 2018; Kolaczkowska & Kubes, 2013; Raziyeva et al., 2021). The role of the neutrophils is to eliminate the possible microbes at the wound site, usually by LTB4 release and NETosis, phagocytosis, ROS production to recruit new immune cells, or presenting antigens to T cells in order to activate adaptative immune response (Kovtun, Messerer, Scharffetter-Kochanek, Huber-Lang, & Ignatius, 2018; Raziyeva et al., 2021).

After the leukocyte recruitment and due to the release of growth factors like PDGF and VEGF, the proliferation phase starts. In this period, new blood vessels and capillaries grow, leading to the recruitment of more leukocytes and the subsequent fibroblast activation leaded by neutrophils, triggering the reepithelization of the tissue (Peiseler & Kubes, 2019; Shim et al., 2022). In a last step, collagen and other ECM components are secreted by fibroblast, allowing the remodeling of the tissue (Gurtner et al., 2008; Ohnstedt et al., 2019; Phillipson & Kubes, 2019).

After tissue repair, neutrophils need to be removed from the skin. At the end of the process, they present in the skin a molecule called phosphatidylserine, a well-known “eat me” signal. This molecule is detected by the CCN1 expressed in the macrophage plasma membrane, triggering the phagocytosis and efferocytosis of the neutrophils, and their following clearance from the affected tissue and leading macrophages to secrete antiinflammatory cytokines like IL-10 or TGFβ (Elliott, Koster, & Murphy, 2017; Ellis et al., 2018; Krzyszczyk, Schloss, Palmer, & Berthiaume, 2018; Lawrence et al., 2020; Phillipson & Kubes, 2019; Raziyeva et al., 2021). Moreover, apoptotic neutrophils release signals like lysophosphatidylserine that activates type 3 ILCs in the skin, involved in tissue repair (X. Wang et al., 2021).

A recent study reported that after a skin wound, recruited and activated neutrophils could reverse migrate to other tissues or organs, such as kidney, carrying out needlessly their effector function and damaging the cells (Skopelja-Gardner et al., 2021). Activated neutrophil-reverse migration studies point out neutrophil clearance as an important mechanism at the end of the inflammatory process (Barkaway et al., 2021; Ji & Fan, 2021; Skopelja-Gardner et al., 2021).

Wounds are not always properly healed by the mechanisms previously described. Although this is not usual, the defective wound healing leads to chronic wounds, usually known as ulcers. The prolonged presence of immune cells is characteristic of chronic wounds, including neutrophils (Ellis et al., 2018; Raziyeva et al., 2021). Ulcers can be associated to bacterial infections and biofilm formation, which could lead to sepsis of amputation (Rahim et al., 2017). The prolonged presence of neutrophils in the skin wound leads to an aberrant function, and excessive NETosis, protease release and ROS production affects the tissue, triggering poor vascularization, tissue fibrosis and a delay in the wound healing process (Raziyeva et al., 2021).

Neutrophils in chronic skin inflammatory disorders

Neutrophils play an important role triggering and aggravating autoinflammatory diseases, like psoriasis, atopic dermatitis or contact dermatitis. That reason makes them an interesting point of attention for new research.

Neutrophils in psoriasis

Psoriatic patients have shown an increase in the Neutrophil-to-Leukocyte ratio (NLR), in the neutrophil activity, and also an increase in the number of immature neutrophils circulating (Carmona-Rivera & Kaplan, 2013; W. M. Wang & Jin, 2020).

In psoriasis, macrophage activation and neutrophil recruitment is triggered by the keratinocyte secretion of IL-17C (IL-25) and autocrine regulation. IL-25-activated keratinocytes overexpress molecules like CXCL8, CXCL10 or CCL5 that trigger neutrophil recruitment and VEGF, promoting angiogenesis and lymphangiogenesis (C. Deng et al., 2021; Lauffer et al., 2020; Senra et al., 2019; Senra et al., 2016; Tan et al., 2013). Recognition of this chemokines by CXCR2 increases the release of LTB4, which acts in an autocrine and paracrine manner being recognized by neutrophil LTB4 Receptor (BLT-1) and amplifying the recruitment (Y. Luo, Luo, Chang, Xiao, & Zhou, 2020; Sumida et al., 2014). A recent study describes that IL-33 and CXCL1 repress an epidermal transcription factor called OVOL1, involved in maintaining the epidermal integrity, enhancing neutrophil accumulation and aggravating skin (Dragan et al., 2022). Sensory nerves in the dermis secrete neuropeptides that activates vasoactive amine release by mast cells, that facilitates neutrophil infiltration (J. E. Choi & Di Nardo, 2018; Siiskonen & Harvima, 2019).

In mice models of psoriasis, some reports described that neutrophil depletion triggers an improvement in the disease (Costa et al., 2022; Han, Havnaer, Lee, Carmichael, & Martinez, 2020; Sumida et al., 2014).

Talking now about their effector functions (**Figure 17**), neutrophils and mast cells have been described as the main IL-17A producers in the skin of psoriatic patients, recruiting and activating

other immune system cells (R. Keijsers et al., 2014; Keijsers, Joosten, van Erp, Koenen, & van de Kerkhof, 2014). Although the mechanism by which neutrophils are activated to produce IL-17A is unknown, the interaction between them and the keratinocyte is a crucial process in this shift (X. T. Liu et al., 2022). Moreover, MMP9 released by neutrophils in psoriasis skin increases vascular endothelial cell activation and permeability, increasing inflammation (Alves-Filho, Marcel Silva Melo, & Ryffel, 2021; J. Chen et al., 2021).

Neutrophils in psoriatic patients show an increased expression of respiratory burst genes like *MPO* and *NOX2*, that leads to an overproduction of ROS in the skin causing an oxidative stress. ROS produced by neutrophils recruited to the tissue activate dendritic cells that lead to trigger T_H response of T cells, stimulating angiogenesis and keratinocyte proliferation in the affected skin (Chiang, Cheng, Korinek, Lin, & Hwang, 2019).

Interaction between activating keratinocytes and neutrophils leads to the release of keratinocyte exosomes and lipocalin 2 (LCN2) that trigger the neutrophil NF-κB-P38 pathway activation, the release of IL-6, CXCL8 and TNFα, and NETosis (M. Jiang et al., 2019; Shao et al., 2019). Psoriatic patients have a higher number of NETotic neutrophils in the skin lesions and in the circualtion, correlating with the severity of the disease, leading to the activation of keratinocyte TLR4 and IL36R crosstalk, increasing the cytokines release by keratinocytes (B. Matta, Battaglia, & Barnes, 2022; Schon, Broekaert, & Erpenbeck, 2017; Shao et al., 2019). The release of NETs activates DCs, which is involved in the activation of T cells and T_H17 responses (Chiang et al., 2019).

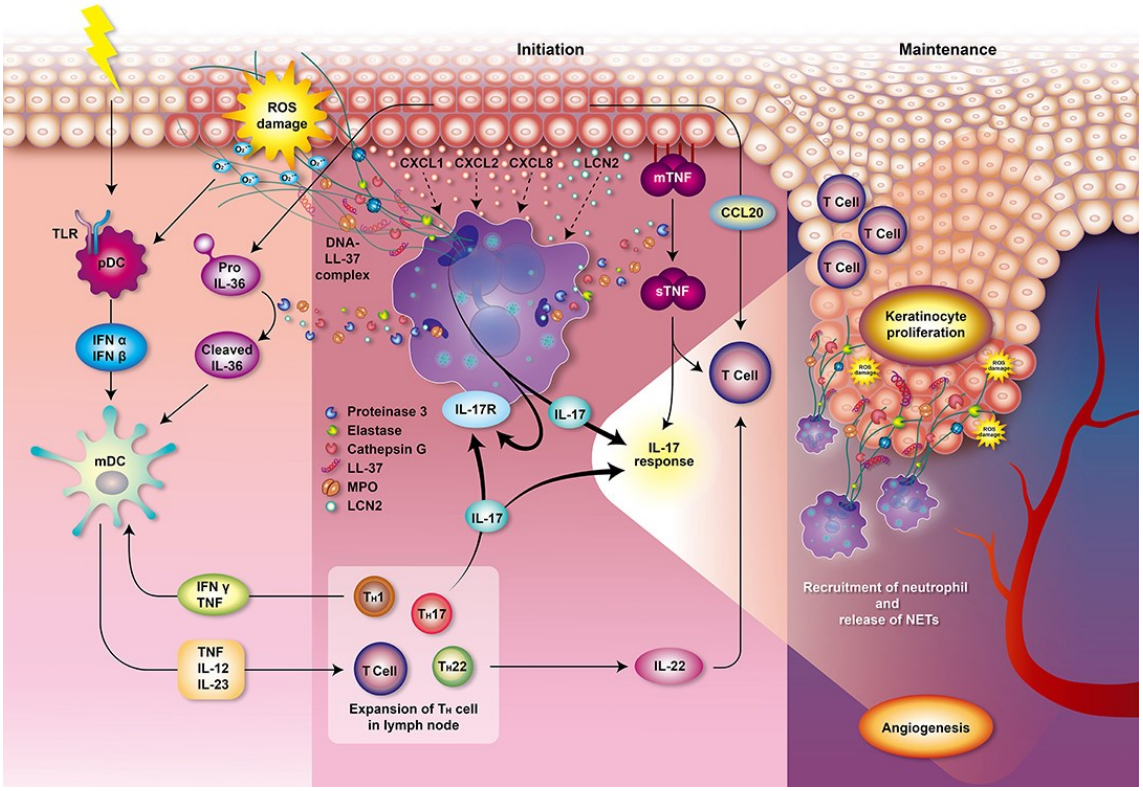

Figure 17. Neutrophil effectors function in psoriasis skin injuries. Different effector functions have been reported for neutrophils in psoriasis. Neutrophils are able to trigger respiratory burst, releasing ROS that can damage keratinocytes during the initiation phase of psoriasis. This ROS are going to activate dendritic cells, releasing cytokines that will activate T cell function. Moreover, neutrophils degranulate releasing proteins like elastase, proteinase 3 or cathepsin G. Finally, neutrophils can also carry out NETosis in the proliferative keratinocytes, releasing their DNA and other protein factors. CCL, CC-chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; IL, interleukin; IFN, interferon; MHC I, major histocompatibility complex class I, MPO, myeloperoxidase; NET, neutrophil extracellular trap; NOX, NADPH oxidase; NE, neutrophil elastase; pDCs, plasmacytoid dendritic cells; mDCs, myeloid dendritic cells; TLR, Toll-like receptor; TNF, tumor necrosis factor (Chiang et al., 2019).

Neutrophils in atopic dermatitis

Although little is known about the role played by neutrophils in atopic dermatitis, recent studies are suggesting their involvement of these leukocytes in the progression of the disease.

The neutrophil-to-lymphocyte ratio correlates significantly with the severity of the disease, being reflected in the higher area of the affecter regions in the skin, and in a higher degree of inflammation in these regions (Dogru & Citli, 2017; Inokuchi-Sakata et al., 2021; Y. Jiang & Ma, 2017). Transcriptomic analyses in skin samples of patients with acute atopic dermatitis showed overexpression of genes related to neutrophilic inflammation, a feature of psoriasis (Choy et al., 2012).

Recent studies showed an important role of neutrophils in atopic dermatitis, related to chronic itch in the lesions, one of the characteristics associated to this disease. The research reported that neutrophil ablation in a mouse model of atopic dermatitis results in an alleviation of this

chronic itch. When neutrophils reached the lesion, an increased hyperinnervation and expression of inflammatory markers and neuropathic itch is seen. The study concluded that neutrophil-derived CXCL10 interacts with sensitive neuron CXCR3, present in the skin, triggering and aggravating chronic itch (Walsh et al., 2019).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and is non canonical roles in inflammation

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most abundant proteins in the cell, commonly consider as a housekeeping gene. This enzyme is really well-known because of its function in glycolysis, but also because of being a moonlighting protein involved in a wide range of non-canonical roles, some of them related to pathological conditions like inflammation or cancer (Lazarev, Guzhova, & Margulis, 2020).

GAPDH structure

GAPDH is a protein of 144 kDa whose structure consists of four identical subunits of 36 kDa which form a tetramer containing 1,332 amino acids, 335 amino-acids per subunit. These subunits form an asymmetric dimer of dimers that results in a homotetramer. Each subunit contains two important domains: an NAD^+-binding domain and a catalytic domain. The NAD^+-binding domain consists of a Rossmann-fold domain in which NAD^+ is able to bind when its conformation is extended and consists on a β-sheet surrounded by two α-helices (Jenkins & Tanner, 2006; Kubo et al., 2016; White et al., 2015). This Rossmann fold is typical of other NAD^+-binding enzymes proteins, like Lactate dehydrogenase (LDH) (Muramatsu et al., 2005). The NAD^+ binding domain also contains phosphatidylserine, glutathione and RNA binding sites (Sirover, 2014). The catalytic domain is formed by a β-sheet flanked on one side by another β-sheet that contacts with the catalytic domain of the next subunit, a structure called “P interface”, and on the other side by three α-helices (**Figure 18**) (Jenkins & Tanner, 2006; Kubo et al., 2016). This enzyme can be found in the cell as a monomer, as a dimer and as a tetramer (Kubo et al., 2016; Nagradova, 2001).

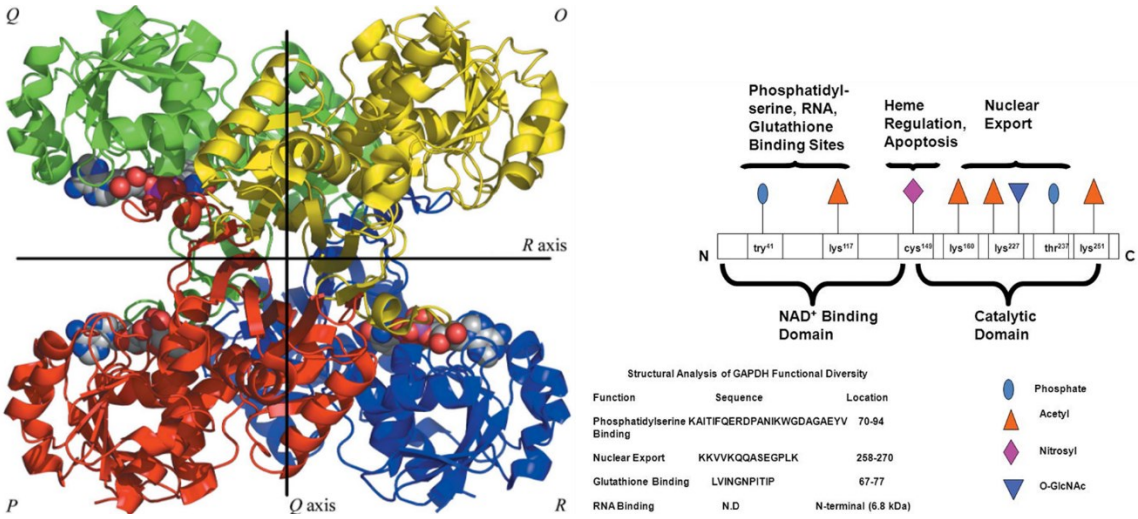

Figure 18. GAPDH structure and domains. GAPDH is an asymmetric dimer of dimers that results in a tetramer. Each monomer on a β-sheet surrounded by two α-helices that conforms the Rossman fold domain, where the NAD^+ cofactors are represented in space filling format, and a β-sheet flanked on one side by another β-sheet and three α-helices that form the catalytic domain. The catalytic domain of each subunit is connected to the next one by the P interface. The NAD^+ binding domain contain the sites responsible of the binding to phosphatidylserine, RNA or glutathione. The catalytic domain contains the sites involved in nuclear export. Finally, between both domains the heme regulation and apoptosis-involved site can be found (Adapted from Sirover et al., 2014 and Jenkins & Tanner, 2006).

GAPDH glycolytic function

The tetrameric conformation of GAPDH allows it to carry out its main and well-known function: glycolysis. The enzyme catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate (Bruns & Gerald, 1976). This reaction is reversible and uses NAD^+ as an electron acceptor, being transformed into NADH, and an inorganic phosphate (**Figure 19**). Two residues in the catalytic domain of each subunit have been reported as crucial to this function, the Cys^{152} and the His^{179}. This GAPDH role is crucial for the production of ATP and pyruvate in the cells (Nicholls, Li, & Liu, 2012).

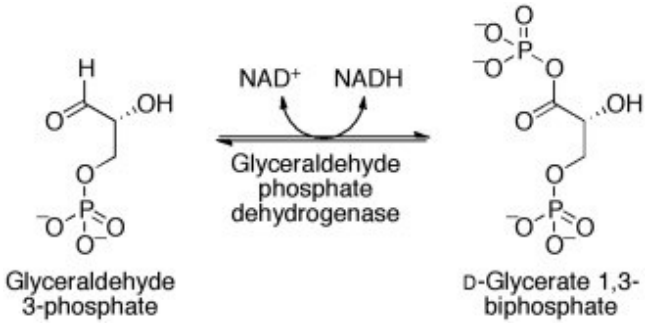

Figure 19. GAPDH role in glycolysis. GAPDH catalyzes the reversible transformation of Glyceraldehyde 3-phosphate into 1-3 Biphosphoglycerate. The reaction requires the reduction of NAD^+ into NADH (Dyer, Ford, & Robinson, 2019).

GAPDH posttranslational modifications

Nitrosylation

In oxidative stress conditions, NOS-derived NO triggers the nitrosylation of GAPDH on the side chain of its active site residue, the Cys^{152}, generating an SNO-GAPDH. Although there is not clear information about the structural changes that nitrosylation triggers in GAPDH, it is well-known that this PTM induces a modification in the structure of the enzyme that allows it to interact with an E3 ubiquitin ligase called SIAH1. The complex formed with SNO-GAPDH-SIAH1 translocates to the nucleus due to SIAH1 nuclear localization sequence (NLS) (**Figure 20**) (Kornberg et al., 2010; Muronetz, Medvedeva, Sevostyanova, & Schmalhausen, 2021; Sirover, 2021).

GAPDH needs SIAH1 to move from the cytosol to the nucleus. This GAPDH-SIAH1 interaction can be interrupted by GOSPEL (GAPDH's competitor *o*f SIAH Protein Enhances Life), a cytosolic protein that competes with SIAH1 and binds GAPDH retaining it in the nucleus (Gonzalez et al., 2016; N. Sen et al., 2009). Although it was reported that GAPDH has a potential NLS (KKVVK) in its catalytic domain, particularly in the residues responsible of the G3P binding (Sirover, 1999), no studies have demonstrated that GAPDH can translocate to the nucleus by itself (Sirover, 2014). In contrast, GAPDH contains in its binding site a nuclear export signal (NES) (KKVVKQQASEGPLK) and mutations in the Lys^{259} inhibit the export, which demonstrate that, although GAPDH cannot be translocated to the nucleus on its own, it can be transported out of the nucleus without any other transporter enzyme (Brown et al., 2004; Sirover, 2014). GAPDH is also involved in binding and nuclear transport of other proteins like nicotinamide phosphoribosyltransferase (NAMPT), sustaining NAD^+ metabolism in the nucleus (Grolla et al., 2020).

Once in the nucleus, a wide number of SNO-GAPDH roles have been described. As the GAPDH nitrosylation is a reversible process, SNO-GAPDH is able to transfer its nitrosyl group to target proteins, acting as a nitrosylase and changing their activity (Kornberg et al., 2010; Sirover, 2021). Posttranslational modifications of other enzymes, heme metabolism and cell death induction are some roles in which SNO-GAPDH is involved.

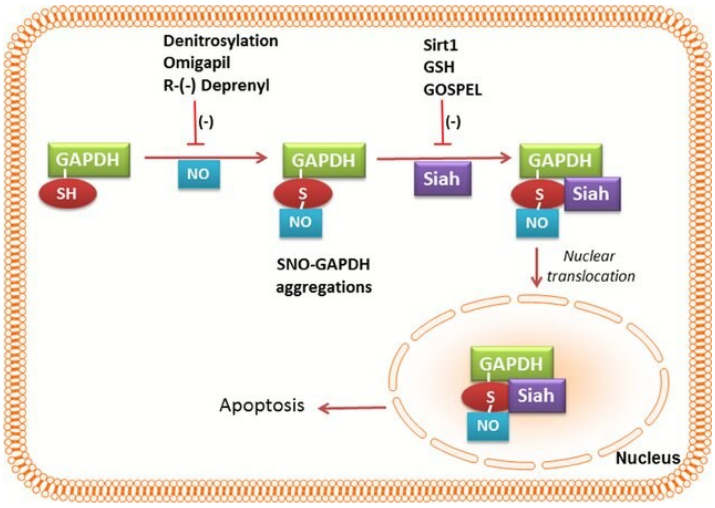

Figure 20. GAPDH nitrosylation and nuclear translocation. Nitric oxide produced in oxidative and nitrosative stress conditions nitrosylate GAPDH in the -SH group of the residue Cys^{152}, in a process called S-nitrosylation. This nitrosylation can be inhibited by denitrosylation or drugs like omigapil or R-Deprenyl. After S-nitrosylation, GAPDH suffers a conformational change that allows the interaction with SIAH1, that can be inhibited by GOSPEL, reduced glutathione or SIRT1. GAPDH-SIAH1 complex is translocated to the nucleus, where GAPDH can trigger functions like cell death. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase, SNO; S-Nitrosylated; GSH, reduced glutathione; SIAH1, Siah E3 Ubiquitin Protein Ligase 1; NO, Nitrix Oxide; Sirt1, Sirtuin 1; GOSPEL, GAPDH's competitor of SIAH Protein Enhances Life. (Dyer et al., 2019)

Acetylation

The acetylation consists of the addition of an acetyl group from a donor, normally acetyl-coA, to specific amino-acids (generally lysines) of certain proteins like histones. This process is typically catalyzed by acetyltransferases. GAPDH is susceptible to acetylation in several lysine residues by P300/CBP-associated factor (PCAF) complex (**Figure 21**) (Sirover, 2014, 2021; Tristan, Shahani, Sedlak, & Sawa, 2011). In particular, GAPDH Lys^{254} acetylation leads to an activation of its glycolytic function in response to an increase in the glucose levels in the cell (T. Li et al., 2014). Moreover, GAPDH acetylation is important in the nuclear translocation process. Acetylation in the residues Lys^{117}, Lys^{227} and Lys^{251} is crucial for the translocation. Also, acetylation in the Lys^{160} is related to apoptotic gene expression (Sirover, 2014, 2021; Ventura et al., 2010).

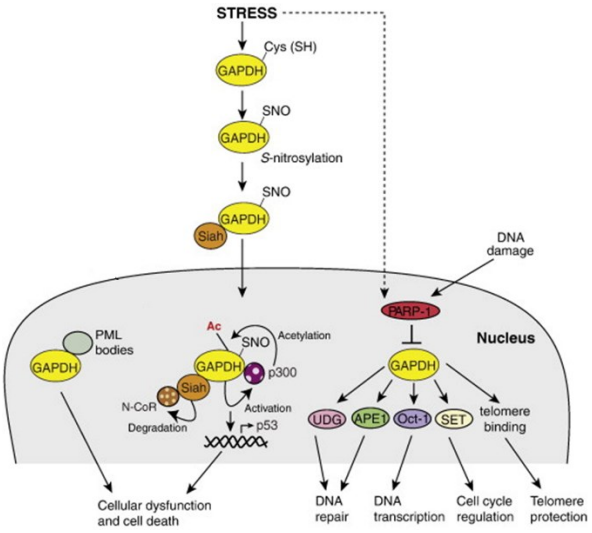

Figure 21. P300/CBP-mediated GAPDH acetylation. After GAPDH nuclear translocation, nuclear GAPDH is able to interact with P300/CBP complex, activating acetylation. One of the first protein targets of P300/CBP is GAPDH, with is acetylated in several lysine residues. After the acetylation, GAPDH-P300/CBP complex is able to activate the expression of genes like *TP53*, regulating functions like cell death or cellular dysfunction. Other functions that GAPDH can carry out in the nucleus are PML bodies binding or PARP-1 interaction. GAPDH, Glyceraldehyde 3-phosphate Dehydrogenase; SIAH1, Siah E3 Ubiquitin Protein Ligase 1, SNO, S-Nitrosylation; PML, Promyelocytic leukemia nuclear bodies; PARP-1, Poly(ADP-Ribose) Polymerase; UDG, uracil-DNA glycosylase; APE-1, Human AP endonuclease; SET, SET Nuclear Proto-Oncogene. Adapted from (Tristan et al., 2011).

Phosphorylation

Phosphorylation consists of the kinase-mediated addition of a phosphate group to the side chain of an amino-acid. GAPDH suffers two phosphorylations in different residues leaded by different kinases. First, Protein Kinase Cίλ carries out a phosphorylation in a still unknown serine residue. The second phosphorylation is carried out by SRC kinase in the residue Tyr^{41} (Sirover, 2021; Tisdale, 2003; Tisdale & Artalejo, 2006, 2007).

These two phosphorylations are sequential and allows GAPDH to participate in the membrane trafficking. Particularly, in the Endoplasmic Reticulum (RE) to Golgi apparatus transport, Rab2 GTPase was reported to be involved in the formation of a pre-Golgi vesicle called vesicular tubular clusters (VTCs) and GAPDH was found as a component of the complex with Rab2 GTPase. In cells treated with an anti-GAPDH antibody, the trafficking is interrupted (Sirover, 2021; Tisdale, 2003; Tisdale & Artalejo, 2006, 2007; Tisdale, Talati, Artalejo, & Shisheva, 2016).

Phosphorylated GAPDH can also act as a kinase, phosphorylating other target residues. This function is important on mediating synaptic transmission. Phospho-GAPDH can phosphorylate the residues Thr^{337} and Ser^{416} of the $GABA_A$ receptor, stimulating its activity (Laschet et al., 2004; Sirover, 2021).

Moreover, the Akt serine/threonine kinase, also known as protein kinase B (PKB), involved in cell proliferation, is able to phosphorylate GAPDH in its Thr^{237}. This phosphorylation prevents SIAH1 binding to SNO-GAPDH, and has been described as a mechanism to avoid SNO-GAPDH-mediated cell death and promote cell proliferation (Q. Huang et al., 2011; Sirover, 2014, 2021)

Finally, GAPDH has been reported as an AMP-activated protein kinase (AMPK) phosphorylation target. Under glucose cell starvation, AMPK is activated and phosphorylates GAPDH on the Ser^{122} residue. This PTM triggers GAPDH nuclear translocation, and its interaction and activation of Sirtuin 1 (Sirt1), leading to cell autophagy (**Figure 22**) (Chang et al., 2015).

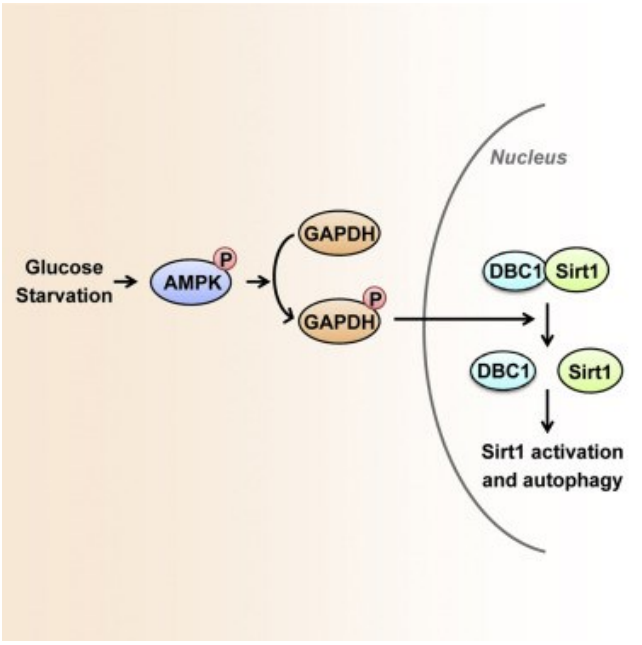

Figure 22. AMPK-mediated GAPDH phosphorylation. Under glucose starvation conditions, GAPDH is phosphorylated by AMPK in the Ser^{122}, which leads to its nuclear translocation and the SIRT1 activation, leading to autophagy. AMPK, AMP-dependent Kinase; GAPDH, Glyceraldehyde 3-phosphate Dehydrogenase; DBC1, Deleted in Breast Cancer 1; SIRT1, Sirtuin 1 (Chang et al., 2015).

Methylation

The methylation consists of the transference of a methyl group to the side chain of an arginine residue. The enzyme Protein Arginine Methyltransferase 1 (PRMT1) can methylate GAPDH in the Arg^{234} residue, preventing the SNO-GAPDH-Mediated cell death in macrophages (Cho, Lee, Kim, Choi, & Choi, 2018). In addition, Arg^{234} methylation can be triggered also by coactivator-associated arginine methyltransferase 1 (CARM1 or PRMT4), suppressing the glycolytic function of GAPDH in liver cancer (Zhong et al., 2018). Another methyltransferase, PRMT3, also methylates GAPDH in the Arg^{248} residue in pancreatic cancer conditions, and in this case this modification activates the glycolytic function of the enzyme (M. C. Hsu et al., 2019).

O-linked N-acetyl glucosamine (O-GlcNac) modification

O-GlcNAcylation is a PTM considered to alter protein nuclear transportation. GAPDH can suffer O-GlcNacylation in its Thr^{227} residue, interrupting the hydrophobic interactions between subunits and disrupting tetrameric conformation of GAPDH. Moreover, O-GlcNac-GAPDH has been found more abundantly in the nucleus, suggesting that this modification can also altered the protein localization (J. Park, Han, Kim, Kang, & Kim, 2009; Sirover, 2014, 2021).

Oxidation

Apart from nitrosylation, oxidative stress can induce oxidation in residues of the proteins, generally in the side chain of cysteines, affecting its thiol group. In GAPDH, it has been reported that thiol oxidation leads to a S-sulfenylation (R-SOH) and then to S-glutathionylation (R-SSG), resulting in a GAPDH reversibly inactive (Talwar et al., 2023). GAPDH presents an H_2O_2 binding side next to the G3P binding site that facilitates the oxidation by a specific mechanism (Peralta et al., 2015; Talwar et al., 2023). The residue oxidated in GAPDH is the Cys^{149}, in the catalytic site (Arutyunova, Danshina, Domnina, Pleten, & Muronetz, 2003; Sirover, 2021). GAPDH oxidation has emerged as a relevant point mediating cellular metabolism. GAPDH oxidative inactivation triggers the transition from glycolysis to pentose phosphate pathway and increase the reductive capacity of the cell by generating NADH. This GAPDH-mediated redox switch allows the survival of stressed cells (Talwar et al., 2023).

Succination

A recent study reported a new PTM that affects GAPDH, succination. The process consists of a covalent modification of the side chain of a cysteine residue by molecules like dimethyl fumarate and monomethyl fumarate. This succination, again, affects to the Cys^{152} residue of GAPDH inactivating its glycolytic function and downregulating glycolysis in myeloid and lymphoid cells, triggering anti-inflammatory effects (**Figure 23**) (Blatnik, Frizzell, Thorpe, & Baynes, 2008; Kornberg et al., 2018; Morozzi et al., 2022; J. B. Park, Park, Son, Ha, & Cho, 2019). This data has leaded to the use of DMF and MMF to treat autoimmune disease and cancer (Lei et al., 2022).

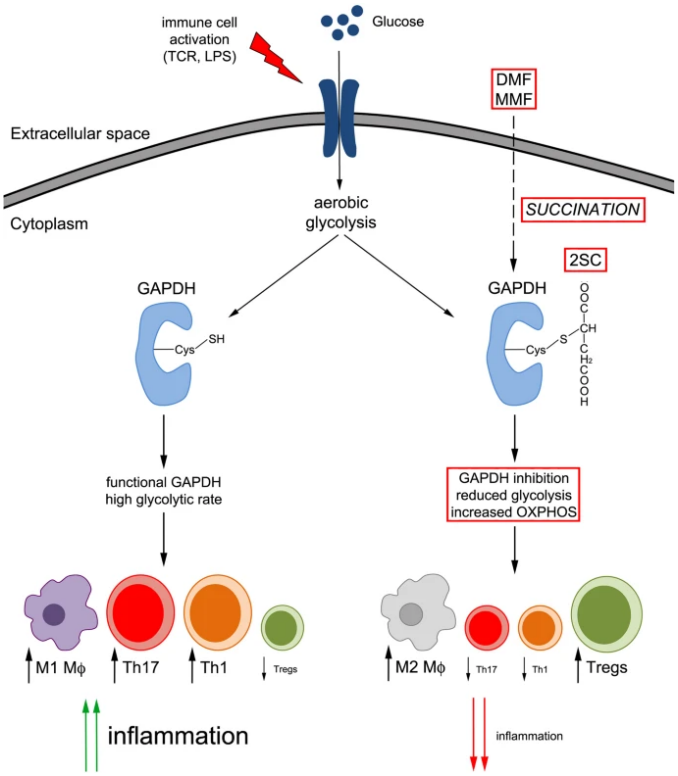

Figure 23. Fumarate-mediated GAPDH succination. Monomethyl and dimethyl fumarate trigger GAPDH succination. In normal conditions, GAPDH does its glycolytic function, immunomodulating the immune system cells to a pro-inflammatory state. However, succination inhibits GAPDH glycolytic function and promotes an anti-inflammatory activity. GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; Tregs, T regulatory cells; TCR, T Cell Receptor; LPS, Lipopolysaccharide; MMF, Monomethyl Fumarate; DMF, Dimethyl Fumarate; Mϕ, Macrophages (Lei et al., 2022).

Non canonical roles of GAPDH

Although GAPDH was commonly known as a housekeeping protein just involved in glycolysis, in the middle of the 1990s, researchers started to report new roles of this enzyme in several pathways, participating in multiple and crucial processes like cell death or gene transcription (Nicholls, Li, et al., 2012). The diversity shown in terms of function is extremely related to its diversity in terms of PTMs (Garcin, 2019; Nicholls, Li, et al., 2012; Sirover, 2021; Tristan et al., 2011).

Cell death function

The role of GAPDH in cell death is one of its most studied extracurricular roles. The main process that triggers all the cascade is the GAPDH S-nitrosylation to SNO-GAPDH in an oxidative context and its binding to SIAH1, leading to nuclear translocation (Hara et al., 2005). Once in the nucleus, GAPDH can trigger different processes that lead to cell death. First, SNO-GAPDH activates PCAF complex, leading to an increase in the expression of genes such as *TP53*. Consequently, P53 activation induces the expression of genes like *BAX*, *PUMA* or *CDKN1A* (P*21*), regulating cell death (Lazarev et al., 2020; N. Sen et al., 2008).

The interaction between SNO-GAPDH and SIAH1 stabilizes both proteins, being unable to be degraded and allowing them to carry out new functions in the nucleus. This S-nitrosylation of SNO-GAPDH is reversible, allowing GAPDH to transnitrosylate other proteins in the nucleus, modifying their functions (Fig. 20) (Kornberg et al., 2010). Among the reported GAPDH transnitrosylation targets, some of the most important are DNA-activated protein kinase (DNA-PK), SIRT1 and histone deacetylase-2 (HDAC2) (Kornberg et al., 2010; Muronetz et al., 2021). In both cases, GAPDH-mediated SIRT1 nitrosylation leads to an increase in the acetylation process, that in case of SIRT1 have been related to Alzheimer Disease by an increase in the SIRT1-mediated acetylation of the Microtubule-associated protein Tau (Muronetz et al., 2021; Nott, Watson, Robinson, Crepaldi, & Riccio, 2008; T. Sen, Saha, & Sen, 2018; J. Y. Zhang et al., 2015).

Nuclear SNO-GAPDH is also able to interact with PARP1 protein, an enzyme involved in the regulation of single strand breaks of DNA by modification of the activity of its targets through a PTM called poly ADP-ribosylation (PARylation). PARylation consists on the addition of ADP-ribose to the side chain of amino-acids (Ray Chaudhuri & Nussenzweig, 2017). Some studies described that excessive oxidative stress triggers extensive DNA damage, triggering a chaos of PARP1 that results in a cell death process known as parthanatos (Fatokun, Dawson, & Dawson, 2014). In these conditions, SNO-GAPDH has been suggested as a potential player of the pathway, interacting with PARP1 and trigger its overactivation (Nakajima et al., 2015).

In cardiomyocyte, GAPDH interacts with the kinase MST1 triggering both GAPDH phosphorylation and MST1 activation, which leads to cardiomyocyte cell death (You et al., 2013).

Finally, GAPDH is able to induce cell death by the interaction with mitochondrial membrane proteins. GAPDH interacts with the voltage-dependent anion channel 1 (VDAC1) triggering the appearance of pores in the membrane and leading to the depolarization and mitophagy (Lazarev et al., 2020; Tarze et al., 2007). Phosphorylation of GAPDH in the Thr^{246} by PKCδ prevents this mitophagy (Yogalingam, Hwang, Ferreira, & Mochly-Rosen, 2013).

GAPDH as a transcription factor

Regulation of gene expression is another extracurricular role in which GAPDH is involved. As discussed above, SNO-GAPDH interacts and activates P300/CBP complex promoting its acetylation of target proteins and histones and regulating epigenetic changes that affect the expression of genes involved in cell death like *TP53* (Fig. 21) (Lazarev et al., 2020; N. Sen et al., 2008).

Moreover, some reports have pointed out GAPDH as a direct regulator of gene transcription. The enzyme interacts with Oct-1, being part of the OCA-S complex, involved in the transcription of S-

phase genes, crucial for cell proliferation (Colell, Green, & Ricci, 2009; Nicholls, Li, et al., 2012; Zheng, Roeder, & Luo, 2003). GAPDH also interacts with the androgen receptor activating its transcriptional factor function (Harada et al., 2007; Kosova, Khodyreva, & Lavrik, 2017).

GAPDH as an RNA binding

GAPDH is also described as a non-canonical RNA Binding Protein (RBP). RBPs are enzymes which bind specific cis-acting sequence motifs in the mRNA and regulate their stability or translation rate. GAPDH is a non-canonical RBP because the RNA binding is just one of its moonlighting functions. The enzyme recognizes 3'-UTR RNA motifs called Adenine-Uridine rich elements (AREs), whose sequence is AU_nA, being n = 3 the most usual (Garcin, 2019). Although it remains controversial, several reports suggest that the RNA binding domain in GAPDH is the Rossmann Fold domain, responsible for the NAD^+ binding (Nagy & Rigby, 1995).

Among the mRNAs targets of GAPDH described until today, we can find interferon-γ (*IFNG*), *c-MYC*, *GMCSF*, *IL-2*, colony stimulating factor-1 (*CSF-1*), cyclooxygenase-2 (*COX-2*), connective tissue growth factor (*CTGF/CCN-2*), endothelin-1 (*ET-1*), angiotensin II type I receptor (*AT1R*), *TNFA*, voltage gated sodium channel type 1α subunit, voltage gated sodium channel type 3α subunit, hypoxia inducible factor 1α (*HIF-1A*), glucose transporter 1 (*GLUT-1*) and RNA component of Telomerase (*TERC*) (Garcin, 2019; P. Liu, Zhong, Cao, Sheng, & Huang, 2020; Nicholls, Pinto, et al., 2012; White & Garcin, 2016; Y. Zhou et al., 2008).

Three different functions of GAPDH after binding to mRNAs have been described (**Figure 24**). The enzyme can stabilize mRNA, increasing the levels of the mRNA of genes like *GLUT-1* in low glucose conditions (McGowan & Pekala, 1996); destabilize mRNAs, decreasing the transcript levels of genes like *SCN1A*, preventing pathologies like Dravet Syndrome (Zeng et al., 2014); or repress ribosomal translation of mRNAs like *IL-2* or *IFNG* (Garcin, 2019; White & Garcin, 2016). The GAPDH mRNA binding function is also implicated in ovarian cancer progression (K. Li et al., 2020; Y. Zhou et al., 2008).

Apart from mRNA, GAPDH was proposed to bind tRNA, with a putative tRNA binding site in the residues 303-308 (Carmona, Rodriguez-Casado, & Molina, 1999; Garcin, 2019; White & Garcin, 2016).

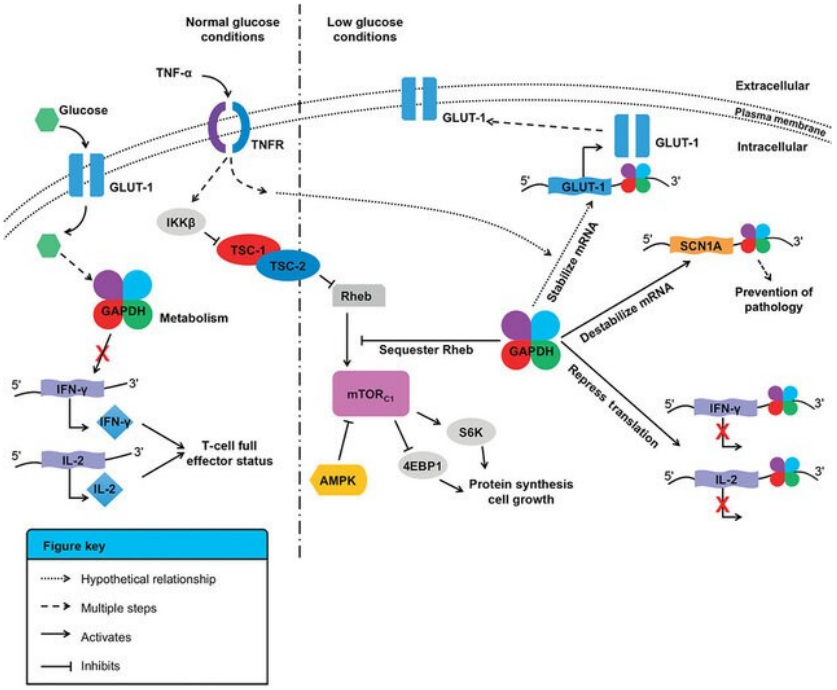

Figure 24. GAPDH RNA binding function. Under normal glucose conditions, GAPDH is involved on its canonical glycolytic function, meanwhile genes like *IFNG* or *IL-2* are being normally expressed and translated. Under low glucose conditions, GAPDH is not carrying out its normal role and their non-canonical role are promoted. GAPDH can sequester Rheb inhibiting mTOR function and cell growth, and it can also bind the AU-Rich regions in the 3'-UTR of different transcripts stabilizing them, like *GLUT-1*, destabilizing, like *SCN1A*, or repressing ribosome binding and subsequent translation, like *IFNG* or *IL-2*. GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; *GLUT-1*; Glucose Transporter 1; *IFNG*, Interferon Gamma; IL-2, Interleukin-2; TNFα, Tumor Necrosis Factor α; TNFR; TNF Receptor, IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; TSC, Tuberous sclerosis 1; AMPK, AMP Kinase; RHEB, Ras homolog enriched in brain; mTOR, mammalian target of rapamycin; 4EBP1, Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; S6K, Ribosomal protein S6 kinase beta-1; *SCN1A*, Sodium Voltage-Gated Channel Alpha Subunit 1 (White & Garcin, 2016).

GAPDH in DNA Repair

One of the roles of SNO-GAPDH after its nuclear translocation is to mediate the DNA repair. Several ways to participate in this process have been reported (Kosova et al., 2017). Among them, GAPDH recognizes apurinic/apyrimidinic (AP) sites in the DNA, one of the most frequent damages of DNA, and nucleotide analogs, interacting with them by its Rossmann Fold domain. This interaction promotes DNA repair by activation of AP endonuclease 1 (APE1), High Mobility Group B1 (HMGB1) and B2 and PARP1, but in conditions of damage accumulation in the DNA, GAPDH forms an irreversible complex with DNA, overactivates PARP1 and promotes parthanatos cell death. (Azam et al., 2008; Fatokun et al., 2014; Kosova, Khodyreva, & Lavrik, 2015; Kosova et al., 2017; Krynetski, Krynetskaia, Bianchi, & Evans, 2003; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021).

GAPDH and telomerase

The relationship between GAPDH and telomerase is polarized. On the one hand, nuclear GAPDH is able to interact with telomeres protecting them by preventing their shortening and degradation. Residues Cys^{149} and Asp^{32} are crucial to carry out this protection. On the other hand, GAPDH interaction with *TERC* inhibits telomerase activity, leading to telomer shortening and cell senescence (Nicholls, Pinto, et al., 2012).

GAPDH in heme metabolism

Recent studies on GAPDH report evidence for a role of the enzyme in heme insertion and metabolism, modulating the activities of hemoproteins as a heme chaperone. This role was firstly described with iNOS. GAPDH is able to bind heme groups and transfer it to iNOS, activating the production of ROS and regulating activities such as immune response (Chakravarti, Aulak, Fox, & Stuehr, 2010). More recent studies have shown that GAPDH also transfers a heme group to guanylyl cyclase and heme oxygenase 2 (HO2) (Dai et al., 2022; Dai, Sweeny, Schlanger, Ghosh, & Stuehr, 2020). This functions also leads GAPDH to play an important role in the maturation of myoglobin and hemoglobin (Tupta et al., 2022).

GAPDH in glucose availability and growth signaling

Another role in which GAPDH is implicated is linking the glucose levels in the cell with the proliferation process carried out by the mTOR complex. In high glucose conditions, there is an increase in the G3P levels in the cell, substrate of GAPDH, activating it glycolytic role. In low glucose conditions, GAPDH is able to sequester Rheb, a GTPase with is part of the mTOR complex, sequestering it and inhibiting its function and cell proliferation and growth (**Figure 24**) (M. N. Lee et al., 2009; Nicholls, Li, et al., 2012).

GAPDH in autophagy

Autophagy is an essential mechanism for maintaining the cell homeostasis and remove damaged components and material at the same time it provides energy to create new cellular components. During the process, the portion of cytoplasm damaged containing proteins, organelles, and other components is included in a structure called autophagosome. This autophagosome later fuses to a lysosome forming an autolysosome which breaks down the material into simple molecules and energy that will be reused by the cell (Levine & Kroemer, 2019). GAPDH is involved in autophagy by different functions. GAPDH-mediated SIRT1 acetylation activates SIR1 function, and this enzyme is able to deacetylate the protein Microtubule-associated protein 1A/1B-light chain 3 (LC3) in the nucleus, inducing autophagy (Butera et al., 2019; I. H. Lee et al., 2008). Moreover, GAPDH-mediated Rheb GTPase

sequestering inhibits mTOR and induces autophagy (Butera et al., 2019; M. N. Lee et al., 2009; Nicholls, Li, et al., 2012). This GAPDH-induced autophagy represents a mechanism by which the cell is protected from cell death (Colell et al., 2007).

Other GAPDH roles

Although the most described extracurricular roles of GAPDH have been currently commented, this enzyme is reportedly involved in more cellular functions that are less described and need further research.

GAPDH participates in the iron trafficking acting as a receptor for lactoferrin and transferrin. High concentration of ATP induces the activation of the channel P2X7 Receptor (P2X7R) that triggers the release of GAPDH to the extracellular space. This extracellular GAPDH acts as a carrier in the trafficking of lactoferrin and iron, and also can interact with the cytoskeleton of host immune cells affecting phagocytosis and immune response (Butera et al., 2019; Chauhan et al., 2015; K. A. Seidler & Seidler, 2013; N. W. Seidler, 2013).

Other GAPDH extracurricular roles comprehend interaction with tubulin and actin to mediate actin polymerization, and regulation of the trafficking between ER and Golgi complex (Butera et al., 2019). Moreover, GAPDH secretion by stromal cells may contribute to the inhibition of tumor cell growth (Kawada et al., 2015).

Some of the GAPDH non-canonical roles are summarized in **Figure *25***.

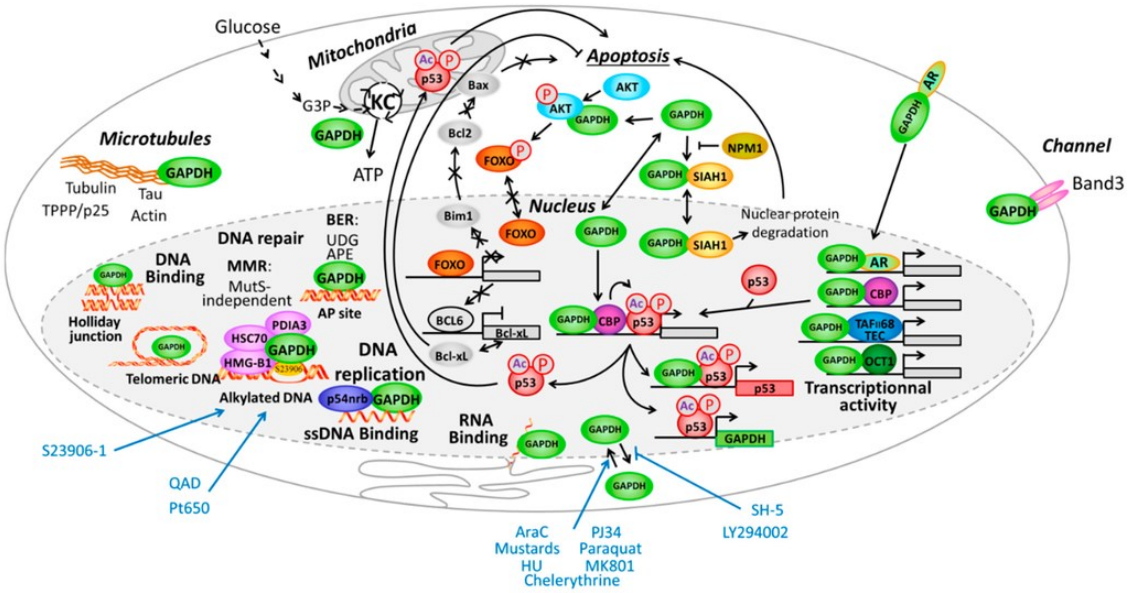

Figure 25. Non-canonical roles of GAPDH. In the figure, different non-canonical roles of GAPDH are represented. Among them, there can be found roles like transcriptional activity, apoptosis, RNA binding, DNA binding, DNA repair, Microtubules organization, DNA replication or membrane transport. GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase, SIAH1, Siah E3 Ubiquitin Protein Ligase 1; AKT, Protein kinase B; NPM1, Nucleophosmin; FOXO, class O of forkhead box transcription factor; CBP, CREB-binding protein; OCT1, POU Domain, Class 2, Transcription Factor 1; BCL, B-cell lymphoma; BAX, Bcl-2 Associated X-protein; PDIA3, Protein disulfide-isomerase A3; HSC70, Heat shock cognate 71 kDa protein; HMG-B1, high mobility group box 1 (Savreux-Lenglet, Depauw, & David-Cordonnier, 2015).

Spermatogenic GAPDH (GAPDHS) or GAPDH-2

In 1992, Welch, J.E. *et al*. reported the expression of a secondary gene of *GAPDH* specifically in the spermatogenic cells of mice. This gene is encoded in a different chromosome and presents an specific amino-acidic sequence in the N-terminus of the protein (Welch, Schatte, O'Brien, & Eddy, 1992). Years later, the same group confirmed the result in human, naming this protein GAPDHS or GAPDH2. (Welch et al., 2000).

GAPDHS is a protein with an identity of 68% with somatic GAPDH and an specific segment of 72 amino-acids in the N-terminus (Welch et al., 2000). In the spermatogenic cells, the enzyme participates in sperm motility and glycolysis, function that carries out more effectively than somatic GAPDH because of the different structure and stability (Elkina et al., 2010; Gill et al., 2022).

Although GAPDHS expression was described as specific in spermatogenic tissue, more recent reports showed that melanoma cells also expressed two isoforms of GAPDHS, the described GAPDHS and a shorter isoform lacking the N-terminal region. The expression of this gene in melanoma suggests an effect increasing metabolism rate, avoiding cell death and metastasis (Gill et al., 2022; Sevostyanova, Kulikova, Kuravsky, Schmalhausen, & Muronetz, 2012).

Roles of GAPDH in inflammatory disorders

The wide variety of functions that can be carried out by GAPDH together with their huge importance, make the enzyme a key character in the regulation of different pathways. Under particular contexts, deregulation of GAPDH function can be associated to the development of diseases. Recently, several studies correlated *GAPDH* mRNA overexpression with the progression of several diseases, like urate crystal inflammation, acute respiratory distress syndrome, pediatric atopic dermatitis, retinal ganglion cells hyper-pressure or atrial fibrillation (AF) following valvular heart disease (VHD) (Della Beffa, Klawonn, Menetski, Schumacher, & Pessler, 2011; Kim, Lee, Seong, Kim, & Lee, 2006; Lavallard et al., 2009; A. Liu, Jia, Liang, & Jin, 2021; Qi, Huang, & Li, 2023; D. Wang, Li, Gu, Liu, & Wang, 2019; L. Wang et al., 2022), and also as a prediction marker in the amniotic fluid of women who suffer preterm birth (Gudicha et al., 2022). Furthermore, *GAPDH* mRNA levels have also been used as a marker of bad prognosis in some type of cancers, like leukemia, esophageal, ovarian, breast, gastric, colorectal or lung cancer (Chiche et al., 2015; Hansen et al., 2009; Hjerpe et al., 2013; Lavallard et al., 2009; Pallares-Rusinol, Moura, Marti, & Pividori, 2023; Peng et al., 2022; Soltany-Rezaee-Rad et al., 2014; Tang et al., 2012; Walter et al., 2016; J. Yang, 2020). A recent study showed that GAPDH is overexpressed in the majority of the different types of tumors whose expression data is kept in The Cancer Genome Atlas, correlating negatively with prognosis and immune tumor infiltration of cells like neutrophils or macrophages (Shen, Li, & Wang, 2023). Although the reason of this GAPDH overproduction remains unknown, a recent paper described BACH1 as a possible regulator (Peng et al., 2022).

These new findings correlating GAPDH functions with disease point out the moonlighting enzyme as a central character of study for the discovery of new pathways and treatments for the different diseases. In this part, GAPDH-mediated pathways involved in disease will be discussed and the controversies highlighted.

In inflammation-induced oxidative conditions, GAPDH is suitable of being modified, affecting its function and leading to a metabolic switch in the cells. The metabolism of immune system is called “immunometabolism”, and studies the role of metabolism in immune cell function, and also the impact of immune cell function in metabolism (Pearce & Pearce, 2013).

Oxidative stress induced by immune system response or by infectious microorganisms is able to affect and compromise GAPDH function. During oxidative stress, GAPDH oxidation and inactivation leads to a pentose phosphate pathway flux that generates NADPH for the cell (Talwar et al., 2023; Torrente & DeNicola, 2023). In a *Mycobacterium tuberculosis* infection, the decrease

in the pH and the release of ROS lead to the oxidation of GAPDH and its inhibition (Gouzy, Healy, Black, Rhee, & Ehrt, 2021). Also, after a neutrophil-mediated immune response, as happens in inflammatory Bowel disease, ROS and RNS produced by the leukocytes damage the colon mucosa, together with the release of NO, generate HOCl and HOSCN that oxidize and inhibit GAPDH (Barrett et al., 2012; McKenzie, Doe, & Buffinton, 1999). This oxidative stress also affects muscle tissue during inflammation-mediated muscle regeneration, affecting metabolic enzymes like GAPDH and creatine kinase (A. P. Pierce, de Waal, McManus, Shireman, & Chaudhuri, 2007), and is observed in other diseases like amyotrophic lateral sclerosis (A. Pierce et al., 2008). A recent study reported that, in COVID-19 patients, immune cells release RNS and S-adenosylmethionine (SAM) produced by RSAD2 trigger GAPDH modification and inhibition (Ebrahimi, Gilbert-Jaramillo, James, & McCullagh, 2021). However, GAPDH is also involved in tissue protection against infection: GAPDH secreted by epithelial cells in response to a *Candida albicans* infection stimulated TLR4 and induced the epithelial secretion of CXCL8 and GM-CSF and the apoptosis of the pathogenic yeast (Wagener et al., 2013).

As discussed above, succination carried out by fumarate and its derivates DMF and MMF in both myeloid and lymphoid cells has been implicated in GAPDH glycolytic function inactivation and polarization to an anti-inflammatory function (Kornberg et al., 2018; Lei et al., 2022; J. B. Park et al., 2019).

Among its non-canonical functions, GAPDH is able to trigger cell death after oxidative stress-induced nuclear translocation in neurons, thymocytes, kidney cells and lymphoma cells (Hara et al., 2005; K. Li et al., 2020; Sawa, Khan, Hester, & Snyder, 1997; N. Sen et al., 2008; Zhai et al., 2015). Despite this well-known role, other study described an anti-apoptotic role of GAPDH in tumor cells (Colell et al., 2007). Moreover, in colon cancer, AKR1B10 was demonstrated to inhibit GAPDH nuclear translocation inhibiting cell death and autophagy (W. Li et al., 2021).

Extracellular GAPDH in infection

One of the most striking roles of GAPDH beyond metabolism is its cell secretion and extracellular function, previously discussed (Butera et al., 2019). Several studies have described GAPDH as a protein commonly secreted by different microorganisms to help during the infection process by interacting with host proteins. Although this secretion is typical in some bacterial infection, a recent study showed that the nematode *Dirofilaria immitis*, responsible of triggering Diofilarasis in dogs, a disease that can be transmitted to humans (Khanmohammadi et al., 2019). Moreover, *Plasmodium* sporozoites, responsible of Malaria transmission, express a GAPDH in the membrane that, after secreting, interacts with CD68 on the Kupffer cell surface and promotes

invasion and disease (Cha, Kim, Pandey, & Jacobs-Lorena, 2016). Other bacteria like *Escherichia coli* also secrete GAPDH during infection (Egea et al., 2007).

Among the different bacteria described to use GAPDH as a pathogenic factor there are different *Streptococcus* (*agalactiae*, *pyogenes* and *pneumoniae*), *Lactobacillus gasseri*, *Mycobacterium tuberculosis*, *Francisella tularensis*, *Bacillus anthracis*, *Brucella abortus*, *Staphylococcus aureus, Leishmania major* or *Listeria sp*. (Alves et al., 2015; Bonifacio Andrade et al., 2022; P. C. Chen et al., 2022; Das, Mukherjee, & Adak, 2021; Fugier et al., 2009; Kopeckova, Pavkova, & Stulik, 2020; Madureira et al., 2011; S. K. Matta, Agarwal, & Bhatnagar, 2010; Oliveira et al., 2012; Pavkova et al., 2017; Teran-Navarro et al., 2021; Terao, Yamaguchi, Hamada, & Kawabata, 2006; Terrasse et al., 2012; Yoshizawa, Yamada, Fujino, & Oda, 2022).

Group B *Streptococcus* GAPDH is one of the most studied. During infection, the secretion of GAPDH by *Streptococcus* induces the production of IL-10, IL-6, IL-1β and TNFα, and inhibits the activity of C5a and the release of H_2O_2 by host cells, suppressing the neutrophil recruitment in mice, facilitating the host colonization (Alves et al., 2015; Madureira et al., 2011; Sullivan et al., 2021; Terao et al., 2006). A recent study suggested that this process can be triggered by GAPDH interaction with TLR2 and TLR4, activating the phosphorylation of MAPKs, PI3K-Akt and NF-κB (Sun et al., 2017). Moreover, the enzyme also induces macrophage inflammatory protein 1 (MIP1), triggering macrophage recruitment and apoptosis in the recruited macrophages in mice (Fettucciari et al., 2000; Oliveira et al., 2012; Sullivan et al., 2021). This data, together with other studies, also suggests that GAPDH could be the responsible of the Group B *Streptococcus*-induced apoptosis of microglia and pulmonary cells during infection (Da Costa et al., 2011; Lehnardt et al., 2007).

Leishmania major is another pathogen recently reported to secrete GAPDH. *L. major* secretes exosomes containing GAPDH, that during infection, enter in macrophages where GAPDH binds to the AU-rich 3'-UTR region of *TNFA* mRNA and inhibits its production, suppressing macrophage-induced inflammation (Das et al., 2021). The commented immunoprotective capacity of secreted GAPDH has also been reported in *B. anthracis* and *L. monocytogenes* (S. K. Matta et al., 2010; Teran-Navarro et al., 2021).

Lactobacillus gasseri also secreted GAPDH that interacts with macrophages inducing a metabolic change that leads to their M1 polarization. The immunometabolic change leads to an attenuation in allergic asthma by preventing the M2 macrophage polarization and the appearance of the Th2-inflammatory microenvironment characteristic of asthma (P. C. Chen et al., 2022). However, GAPDH isolated from *Clostridium difficile* lacks this ability.

In *Francisella tularensis*, secreted GAPDH was related to the replication inside macrophages and to a defective immune response (Pavkova et al., 2017). Moreover, secreted GAPDH is able to interact with fibrinogen, fibronectin and plasminogen, degrading extracellular matrix proteins and facilitating host colonization and infection (Kopeckova et al., 2020; Pavkova et al., 2017).

Some bacteria such as *Mycobacterium tuberculosis* or *Streptococcus pyogenes* export GAPDH to mediate iron acquisition, a crucial process for their survival. Secreted GAPDH interacts with lactoferrin and transferrin and carries iron internalized in phagosomes that are newly trafficked to the bacteria (Chauhan et al., 2015; Malhotra et al., 2017; X. Y. Yang et al., 2016).

Due to the reported roles of extracellular GAPDH during infection, the protein emerges as an antigen and as a potential therapeutical target for new treatments. Nowadays, some researchers have described that GAPDH peptides can be detected by lymphocyte T receptors, which has leaded to the design and development of vaccines against the different microorganisms (Calderon-Gonzalez et al., 2014; Cha et al., 2016; Kopeckova et al., 2020; Perez-Casal & Potter, 2016; Teran-Navarro et al., 2021; S. Yang et al., 2020).

Impact of GAPDH in macrophages

Monocytes and macrophages present an important dependence between metabolism and inflammatory response (Wculek et al., 2023). Inflammatory stimuli in the context of disease, triggers a metabolic switch in this immune cell. On the one hand, pro-inflammatory stimuli are usually related to an M1 polarization that leads to an increase in glycolysis and Warburg effect in the cells. On the other hand, anti-inflammatory conditions cause an M2 polarization and an increase in oxidative phosphorylation (OXPHOS) (Kelly & O'Neill, 2015; Van den Bossche, Baardman, & de Winther, 2015). Due to the fact that GAPDH is one of the key enzymes in glycolytic metabolism, and has emerged as a rate-limiting enzyme in Warburg effect (Liberti et al., 2017), this protein also plays an important role in metabolic reprogramming and linked immunomodulation of macrophages (Iwamoto, Inoue, Tachibana, & Kawahara, 2021; Nakano et al., 2018).

In inflammatory diseases like atherosclerosis, DAMPs released like cholesterol crystals activates macrophages, which show an overexpression of GAPDH, leading to an increased glycolytic metabolism and M1 polarization, characterized by the overexpression of CXCL9 and CXCL10 (O'Rourke et al., 2022). However, other study reporting that extracellular GAPDH secretion by tumor-associated macrophages could lead also to an immunosupresive M2 polarization, suggesting that GAPDH levels are not the only important factor to determine immunomodulation or metabolic switch, probably due to the multiple roles of the enzymes and

also because of the roles of lactate, the product of Warburg effect, in immunomodulation (Nakano et al., 2023; Nakano et al., 2018; Y. Zhang, Zhang, Meng, Xu, & Zuo, 2022).

Some external factors can modify GAPDH structure, modifying its glycolytic role and polarizing macrophages. In oxidative conditions, ROS and RNS induce GAPDH nitrosylation in macrophages, triggering its nuclear translocation and inhibiting its glycolytic role (Messmer & Brune, 1996). To protect GAPDH from RONS-mediated oxidation, monocytes and macrophages use scavengers like glutathione (Y. T. Yang, Whiteman, & Gieseg, 2012). A recent study reported that in low-grade inflammatory conditions, like aging, macrophage metabolic enzyme hexokinase 1 (HK1) is dislocated from the mitochondria and moves to the cytosol where it interacts with S100A8/A9 proteins and trigger iNOS-mediated GAPDH nitrosylation and inhibition, leading to a defective proinflammatory phenotype and cell death (De Jesus et al., 2022). However, oxidative stress context, PRMT1 is able to methylate GAPDH inhibiting its nitrosylation, nuclear translocation, and cell death (Cho et al., 2018). Moreover, methionine sulfoxide reductase B1 (MSRB1) has been described as another important inflammatory regulator by GAPDH oxidation in Met^{44}. In mice, the lack of this protein leads to an increase in glycolysis, excessive pro-inflammatory response and consequent hyper-inflammation (H. J. Yoo et al., 2022).

During inflammatory response, macrophages overexpress Immunoresponsive gene 1 (IRG1), responsible of the catalysis of itaconate, an important metabolite with anti-inflammatory functions (Michelucci et al., 2013; Mills et al., 2018). Recent studies showed that itaconate induces macrophage M2 polarization and anti-inflammatory response inactivating GAPDH glycolytic function by alkylating Cys^{22} (Day & O'Neill, 2022; Liao et al., 2019).

In microglia, GAPDH also regulates neuroinflammation. Excessive ATP production in the central nervous system (CNS) activates P2X7 receptor, which triggers GAPDH release in exosomes and leads to the NLRP3 inflammasome assembly and inflammation (Takenouchi et al., 2015). Moreover, inflammation progression in the CNS leads to a microglia-derived NO overproduction that triggers GAPDH nitrosylation and neuronal cell death in encephalomyelitis (Godfrey et al., 2022).

As discussed above, inflammatory-mediated GAPDH PTMs, apart from inactivating the glycolytic function, promote its non-canonical roles. GAPDH is able to bind mRNA of *TNFA* repressing its translation and reducing inflammation (Millet, Vachharajani, McPhail, Yoza, & McCall, 2016; Xiao et al., 2023). However, in inflammatory conditions, GAPDH is malonylated by acetyl-CoA carboxylase 1 (ACC1) in its Lys^{213}, inactivating its mRNA binding function and promoting expression of inflammatory genes like *TNFA* or *IFNG* (Galvan-Pena et al., 2019; Yamaguchi et al.,

2016). After *IFNG* expression, GAPDH is also part of the IFN-γ-activated inhibitor of translation (GAIT) complex, repressing the translation of target mRNAs like *VEGFA* (Arif, Chatterjee, Moodt, & Fox, 2012). This GAPDH malonylation and following inflammation is disrupted by sodium glucose co-transporter 2 inhibitor (SGLT2i), which inhibits ACC1 (L. Li et al., 2023).

During *Mycobacterium* infection, neurons and astrocytes have been reported to produce hydrogen sulfide (H_2S) that induce protective autophagy in infected macrophages (Kimura, Shibuya, & Kimura, 2012). H_2S mediates GAPDH sulfhydration on $Sser^{122}$, triggering GAPDH translocation to the nucleus, where it inhibits cell cycle activator and apoptosis regulator 2 (CCAR2), activating SIRT1-mediated autophagy (Iqbal, Bajeli, Sahu, Bhat, & Kumar, 2021). Furthermore, GAPDH also migrates to the macrophage plasma membrane, triggering the cellular internalization of the defense peptide LL-37, which activates autophagy and bacterial phagocytosis (Dhiman et al., 2023).

GAPDH also regulates iron trafficking in macrophages, working as a lactoferrin and transferrin receptor upon iron depletion and hypoxia, generating GAPDH-transferrin and GAPDH-lactoferrin complexes in the membrane that are internalize into endosomes (Malhotra et al., 2019; Polati et al., 2012; Raje, Kumar, Harle, Nanda, & Raje, 2007; Rawat, Kumar, Sheokand, Raje, & Raje, 2012). In other species, like bovine, GAPDH carries out macrophage scavenger receptor (MSR) binding and internalization, activating inflammation (Nakamura et al., 2002). This pathway is related to diseases like atherosclerosis (Nakamura et al., 2002).

During macrophage migration in inflammation, GAPDH is able to binds to microtubular plasminogen activating the proteolytic remodeling of the ECM and the macrophage movement (Chauhan et al., 2017).

Impact of GAPDH in neutrophils

Although the different roles of GAPDH in neutrophils have not been as studied as in macrophages, the moonlighting enzyme has been related to different important neutrophil functions. Neutrophils has shown increased GAPDH levels after activation *in vitro* and *in vivo*, in context of diseases like acute respiratory distress syndrome, together with other proteins like MAPK8, MMP9 or PIK3CB, suggesting a role in neutrophil migration (Capuozzo et al., 2017; D. Wang et al., 2019). Treatments with taurine and taurine-derived compounds show a decrease in neutrophil activation and GAPDH levels (Capuozzo et al., 2017). Oxidative molecules like taurine bromamine or taurine chloramine trigger GAPDH oxidation and inactivation in neutrophils (Tokunaga, Kanayama, & Miyamoto, 2007).

In hypoxic conditions, single cell RNA-seq studies with critically-ill COVID-19 patients revealed an increase in HIF-1α, pro-inflammatory and metabolic gene expression, being GAPDH one of them and confirming the correlation between metabolism and immunomodulation in neutrophils, the same way as happens in macrophages (Taniguchi-Ponciano et al., 2021). GAPDH is classically described as a HIF-1α target in hypoxia conditions (Graven, Yu, Pan, Roncarati, & Farber, 1999; S. Lu, Gu, Hoestje, & Epner, 2002), and a study also suggested that this GAPDH overexpression could also be related to the inhibition of cell death and neutrophil survival (Walmsley et al., 2005).

Although there are no recent evidences, in 1995, a study revealed that in circulating neutrophils activated with fMLP, iNOS-derived NO triggered GAPDH ADP-ribosylation (Amin et al., 1995). There is no clear evidence about what ADP-ribosylation of GAPDH in neutrophils is triggering, and further research should be done to elucidates it. More recently, SIAH1-dependent GAPDH nuclear translocation was observed in inflamed neutrophils after spiral cord injury. This pathway has been related to neutrophil apoptosis and inflammation and the study pointed out Neutrophil Elastase (NE) as potential trigger of this process (Huo, Zhu, Ma, Dong, & Su, 2016).

The main GAPDH-related function in neutrophils is NETosis, although the role of the enzyme remains controversial. GAPDH is overexpressed in activated neutrophils carrying out NETs formation, together with other NETosis-related genes like NE or PAD4 (Agraz-Cibrian, Giraldo, & Urcuqui-Inchima, 2019). GAPDH has been classically known as a potent Ca^{2+}-dependent fusogen (similar to annexin I) involved in neutrophil degranulation and possibly in NETs formation and release (Hessler et al., 1998). Moreover, apart from its overexpression, a recent study reported that in human neutrophils suffering extracellular trap formation, GAPDH suffers S-glutathionylation, suggesting that one of its non-canonical roles is taking part in NETosis (Awasthi et al., 2023). In spite of this suggested role of GAPDH in promoting NETosis, a metabolic study in neutrophils of severe COVID-19 patients revealed a decreased glycolytic activity of GAPDH and involvement in NETs formation suppression. Thus, inhibiting GAPDH, NETosis was impaired (Y. Li et al., 2023).

Zebrafish as a model to study inflammatory diseases

The zebrafish.

Zebrafish (*Danio rerio H.*) is a small teleost fish (around 5-6 cm length) included in the *Cyprinidae* family that lives in the rivers of Bangladesh, Northern India and South Nepal (Tonon & Grassi, 2023). Their lifespan is approximately three years in nature, while in laboratory, they can live over than 5 years (Kishi, Slack, Uchiyama, & Zhdanova, 2009). In spite of its classical use as a

developmental model, zebrafish use as a model to study human diseases was first described in 1981 (Streisinger, Walker, Dower, Knauber, & Singer, 1981). Due to its qualities, zebrafish has emerged as an important animal model to model and study human diseases. Large scale genome sequencing experiments comparing human and zebrafish genome showed that, approximately, a 70% of human genes present at least one orthologue in zebrafish, and talking about disease-related genes, the percentage increases to 82% (Howe et al., 2013).

Zebrafish present different advantages regarding the use or other animal models. Its low size allows to keep a lot of individuals in small areas, reducing maintenance and costs (Lieschke & Currie, 2007). Zebrafish generation time is really short, presenting after 24 hours post fertilization (hpf) almost all the precursors for major organs and being completely functional at 48hpf, where hatching happens (Hoo, Kumari, Shaikh, Hue, & Goh, 2016; Tonon & Grassi, 2023). The maturity is achieved as soon as 3 months post fertilization, with female zebrafish being able to spawn more than 200 eggs per week (Hoo et al., 2016; Spence, Gerlach, Lawrence, & Smith, 2008). The generation of a huge number of individuals per week makes zebrafish a great model drug discovery through high-throughput screening, where compounds penetrate the skin after bath immersion (Lubin et al., 2021; Patton, Zon, & Langenau, 2021). The external development and transparency of the eggs facilitate their manipulation, triggering the creation of new genetic technologies than can be easily applied, generating mutant or transgenic zebrafish models with specific conditions that allow the research of particular diseases (Lieschke & Currie, 2007; Spence et al., 2008; Tonon & Grassi, 2023). More recently, gene editing technologies like CRISPR/Cas9 knock-out and knock-in generation, as well as CRISPR/Cas13d RNA editing have emerged as crucial tools to study gene function in diseases using zebrafish as a model of research (Ablain, Durand, Yang, Zhou, & Zon, 2015; Albadri, Del Bene, & Revenu, 2017; Kushawah et al., 2020; Rafferty & Quinn, 2018).

Zebrafish in the study of inflammation.

Zebrafish mutants: the spint1a -/- line

Serine peptidase inhibitor, Kunitz type 1a (Spint1a), also known as Hepatocyte growth factor activator inhibitor 1a (Hai1a), is an important regulator of the activity of Matriptase 1, a transmembrane serin protease responsible of the degradation of the ECM (Carney et al., 2007). By *spint1a* gene disruption, Carney et al. generated a mutant line, the *hi2217*, which displayed basal keratinocytes aggregates and epidermal disruption (Carney et al., 2007). This mutant line also displayed neutrophil dispersion, with the presence of a high number of neutrophils infiltrated in the inflamed skin, and keratinocytes showed to suffer a non-apoptotic cell death (Mathias et al., 2007). Mmp9 was reported as one of the crucial proteins implicated in the

collagen reorganization during repair in this model (LeBert et al., 2015). Moreover, the mutant line showed entosis and apical cell extrusion of the keratinocytes present in the aggregates (Armistead, Hatzold, van Roye, Fahle, & Hammerschmidt, 2020). Recently, another study showed that the Spint1a-deficient line showed oxidative stress in the skin, that triggered excessive DNA damage in the cell that led to a overactivation of PARP1 fueled by NAD^+-derived NAMPT and *parthanatos* cell death *(Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021)*.

Zebrafish transgenic lines for the study of inflammation.

Given the optical transparency of zebrafish embryos and its easy genetic manipulation, the generation of transgenic lines is a crucial technique to allow the *in vivo* visualization of particular cell types or cellular processes like cell cycle (Bouldin & Kimelman, 2014; Poplimont et al., 2020; Renshaw et al., 2006; Sugiyama et al., 2009). Other transgenic lines allow tissue-specific overexpression or depletion of proteins (Iida, Wang, Hondo, & Sehara-Fujisawa, 2020; Langenau et al., 2004). Bio-imaging advances have carried out new non-invasive microscopy techniques for the discovery of different types of functions in zebrafish, highlighting Multi-Photon, Light-Sheet Microscopy and Second Harmonic Generation (Abu-Siniyeh & Al-Zyoud, 2020).

The generation of rerpoter lines is based on the expression of fluorescent proteins regulated by specific promoters of the cell type of interest. According to immune system inflammation, transgenic lines to study neutrophils (Hall, Flores, Storm, Crosier, & Crosier, 2007; Renshaw et al., 2006), macrophages (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011), erythrocytes (Traver et al., 2003), endothelial cells (Lawson & Weinstein, 2002) or T cells (Y. Zhang & Wiest, 2016), among others, have been developed. Also, the generation of inflammation reporters, like *TgBAC*(*tnfa:GFP*), have been really useful for the monitorization and study of the different inflammatory processes (Marjoram et al., 2015).

OBJECTIVES

1. Study the role of GAPDH in chronic skin inflammation, with a focus on the impact of its nuclear translocation, using the Spint1a-deficient zebrafish model and human 3D organotypic skin models of psoriasis.
2. Analyze the gene expression patterns of various cell populations through scRNA-seq and, in particular, characterize the gene expression profiles of peridermal and basal keratinocytes in both wild-type and Spint1a-deficient larvae.
3. Characterize the role played by neutrophils in chronic skin inflammation using the Spint1a-deficient zebrafish model.
4. Evaluate the influence of Gapdh nuclear translocation on neutrophil behavior in the context of chronic skin inflammation using the Spint1a-deficient zebrafish model.
5. Analyze the gene expression profile and signaling pathways in neutrophils regulated by Gapdh nuclear translocation by RNA-seq using the Spint1a-deficient zebrafish model.
6. Study the impact of Gapdh nuclear translocation on neutrophil recruitment in response to acute inflammation.

MATERIALS AND METHODS

· Animals

Wildtype zebrafish (*Danio rerio*) lines AB, TL and WIK were obtained from the Zebrafish International Resource Center (ZIRC). The handling was carried out according to the zebrafish handbook rules (Westerfield, 1993). The different zebrafish transgenic lines used in this project were *Tg(lyz:dsRED)*^{nz50} (Hall et al., 2007), and *Tg(NFkB-RE:eGFP)*^{sh235Tg} (Kanther et al., 2011), *Tg(mpeg1:eGFP)*^{gl22} (Ellett et al., 2011), *Tg(mpx:Gal4.VP16)*^{i222} (Davison et al., 2007), *Tg(UAS:nfsB-mCherry)*^{c264} (Davison et al., 2007), *Tg(lyz:BFP)* (Rosowski et al., 2018), *Tg(lyz:MITO-Dendra2)* (W. Zhou et al., 2018), *Tg(cd41:GFP)* (C. Y. Zhang et al., 2018) and *Tg(gata1a:dsRED)* (Traver et al., 2003). The mutant zebrafish line *Tg(spint1a)*^{hi2217} (*spint1a/hai* mutant for simplicity) (Carney et al., 2007) was provided by Professor Matthias Hammerschmidt. Further information about the different zebrafish lines used can be found in **Table 1**.

Table 1. Zebrafish lines used in this project.

Line name	Description	Reference
Tg(lyz:dsRED)^{nz50}	Neutrophils express the red fluorescent protein dsRED.	(Hall et al., 2007)
Tg(NFkB-RE:eGFP)^{sh235Tg}	NF-κB activity is monitorized due to the expression of GFP.	Kanther et al., 2011
Tg(mpeg1:eGFP)^{gl22}	Macrophages express the green fluorescent protein GFP.	Ellett et al., 2011
Tg(mpx:Gal4.VP16)^{i222}	Neutrophils express the transcription factor Gal4, which recognizes UAS repeats in gene promoters.	Davison et al., 2007
Tg(UAS:nfsB-mCherry)^{c264}	The fish has a transgene which consists on the nitrorreductase joined to the red fluorescent protein mCherry regulated by a UAS-rich region in the promoter	Davison et al., 2007
Tg(lyz:BFP)	Neutrophils express the blue fluorescent protein BFP.	Rosowski et al., 2018
Tg(lyz:MITO-Dendra2)	Neutrophil mitochondria express the photoconvertible protein Dendra2	Zhou et al., 2018
Tg(cd41:GFP)	HSPCs and platelets express the green fluorescent protein GFP.	C. Y. Zhang et al., 2018
Tg(gata1a:dsRED)	Erythrocytes express the red fluorescent protein dsRED.	Traver et al., 2003

Tg(spint1a)^{hi2217}	Neutrophil infiltration, epithelial disruption and keratinocyte hyperproliferation	Carney et al., 2007

· Ethics statement

The experiments performed comply with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Bioethical Committees of the University of Murcia (approval numbers #75/2014, #216/2014 and 395/2017) and Ethical Clinical Research Committee of The University Hospital Virgen de la Arrixaca (approval number #8/13).

· Chemical treatments

Zebrafish embryos were subjected to manually dechorionation at 24 hpf. Larvae were treated from 24 hpf to 48 hpf or 72 hpf, or from 48 hpf to 72 hpf by chemical bath immersion at 28ºC. The incubation was carried out in 6-well plates containing 20 larvae per well in egg water with methylene blue (including 60 µg/mL of sea salts in distilled water). As all the compounds were diluted in dimethyl sulfoxide (DMSO), the control larvae wells were supplemented with 1% DMSO. The different compound used and their characteristics are shown in the **Table 2**.

For neutrophil ablation, larvae *Tg(mpx:Gal4.VP16; UAS:NTR-mCherry)* were treated at 1 dpf with 5 mM Mtz and kept in dark. At 48 hpf the drug was renewed and larvae were treated up to 3 dpf. The larvae were imaged once a day up to 7 dpf and the number of neutrophils determined (Davison et al., 2007; Halpern et al., 2008).

Table 2. Different compounds used in this project.

Name	Description	Model used	Concentration	Reference
CGP3466B (omigapil)	Inhibits GAPDH nuclear translocation by blocking the nitrosylation of the Cys^{152} residue.	Zebrafish, human organotypic 3D models, CD34+ colonies and human isolated neurophils.	10 µM and 1 µM (zebrafish). 1 µM human cells.	SML1941 (Sigma-Aldrich)

Apocynin (Acetovanillone)	Inhibits NADPH oxidase activity.	Zebrafish	250 µM	S2425 (Selleckchem)
SIN-1	Nitric Oxid (NO) donor.	Zebrafish	1 mM	567028 (Sigma-Aldrich)
Flagellin	TLR5 agonist.	Human isolated neutrophils	1 µg/mL	tlrl-epstfla-5
G-CSF	Neutrophil activation.		10 ng/mL	
FMPL	Neutrophil activation.		100 nM	

· Genetic inhibition in zebrafish

The CRISPR RNA (crRNA) obtained from Integrated DNA Technologies (IDT) with the following target sequence were used: *nox1* crRNA: CAAGCTGGTGGCCTACATGA; *nox2 (cybb)* crRNA: CCTCCATGGAGGTCATCCGA; *nox4* crRNA: TTCGCTTGTGTCCTTCAAGC; *nox5* crRNA: GAGGTCATGGAAAATCTCAC; *gapdh* AA crRNA: AAGACCGTTGATGGGCCCTC; *gapdh* AB crRNA: TTCCATGGGTGGAGTCGTAC; *gapdh* AD crRNA: GGACCCAGCCAATATTAAGT; *gapdhs* crRNA: CGGTGGACTCGACCACGTAC; *siah1* crRNA: CCCCTGCAAGTACGCCTCAT. They were resuspended in duplex buffer at 100 µM, and 1 µL was incubated with 1 µL of 100 µM trans-activating CRISPR RNA (tracrRNA) at 95°C for 5 minutes and then 5 minutes at room temperature (RT) to form the complex. Then, the mix was diluted with 1.45 µL of duplex buffer. 1 µL of this complex was mixed with 0.30 µL of recombinant Cas-9 (10 mg/mL), 0.25 µL of phenol red, and 2.55 µL of duplex buffer. When two crRNAs were microinjected at the same time, they were incubated with the tracrRNA independently, and 1 µL of each complex was mixed with 0.30 µL of recombinant Cas-9 (10 mg/mL), 0.25 µL of phenol red, and 1.55 µL of duplex buffer. The efficiency of each crRNA was determined by the ICE webtool from Synthego (https://ice.synthego.com/#/) (Conant et al., 2022). In the case of the *gapdh* and *gapdhs* crRNAs, due to issues in the sequencing, the efficiency was determined by western blot, qPCR and GAPDH activity kit. Crispant larvae of 3 dpf were used in all studies.

· DNA Construct and Generation of Transgenics

The *lyz:gapdh*, *lyz:gapdh*^{C150S}, *runx1:gapdh* and *runx1:gapdh*^{C150S} constructs were generated by MultiSite Gateway assemblies using LR Clonase II Plus (Life Technologies) according to standard

protocols and using Tol2kit vectors described previously (Kwan et al., 2007). The corresponding transgenic lines were generated microinjecting 1nl into the yolk sac of one-cell-stage embryos a solution containing 38.5 ng/µl of the different plasmids, 50ng/µL of transposase RNA, and 0.05% phenol red solution using a microinjector (Narishige).

· Morpholino, RNA and Protein Injection in zebrafish

Specific *gata1a* morpholino (Gene Tools) was resuspended in nuclease-free water at 1 mM. In vitro-transcribed RNA was obtained following the manufacturer's instructions (mMESSAGE mMACHINE kit, Ambion). Morpholinos and RNA were mixed in microinjection buffer and microinjected into the yolk sac of one-cell-stage embryos using a microinjector (Narishige) (0.5-1 nL per embryo). The same amount of MOs and/or RNA was used in all experimental groups.

· Zebrafish larvae imaging

Forty eight and 72 hpf larvae were anesthetized using buffered tricaine at 200 µg/mL dissolved in egg water supplemented with methylene blue. Images were captured with an epifluorescence LEICA MZ16FA stereomicroscope set up with green and red fluorescent filters. All images were acquired with the integrated camera on the stereomicroscope and were analyzed to determine number of neutrophils and their distribution in the larvae with the zebrafish line *Tg(lyz:dsRED2)*. The transcriptional activity of NF-κB was visualized and measured with the zebrafish line *Tg(NFκB-RE:eGFP)*.

· Quantification of the hydrogen peroxide levels.

H_2O_2 release was quantified employing the live cell fluorogenic substrate acetyl-pentafluorobenzene sulphonyl fluorescein (Cayman Chemical, Ann Arbor, Michigan, USA). Briefly, about 20 embryos of 72 hpf were rinsed with egg water and collected in a well of a 24-well plate with 50 µM of the substrate in 1% DMSO for 1 hour (de Oliveira et al., 2015). ImageJ software was employed to determine mean intensity fluorescence of a common region of interest (ROI) placed in the dorsal fin for H_2O_2 production quantification. Similarly, a ROI located in muscle or skin was used to obtain mean intensity fluorescence of *Tg(NFκB-RE:eGFP)* transgenic line.

· WIHC in zebrafish

EdU incorporation assay was used to determine cell proliferation. Embryos of 48 hpf were incubated in 10 mM of EdU dissolved in egg water for 6 hours at 28°C followed by a 1-hour wash out with egg water and fixation in 4% paraformaldehyde (PFA) with PIPES overnight at 4°C or 2 hours at RT. For the rest of immunofluorescence techniques, embryos/larvae were directly fixed

in 4% paraformaldehyde (PFA). Embryos/larvae were then washed with phosphate buffer saline (PBS) with 0.1% tween-20 (PBST) 3 times for 5 minutes. In order to dehydrate the sample progressively, 25%/75% methanol (MeOH)/PBST, 50%/50% MeOH/PBST and 75%/25% MeOH/PBST and 100% MeOH were employed each for 5 minutes. At this point, embryos were stored at −20°C. To proceed with the immunofluorescence, samples were re-hydrated in decreasing solutions of MeOH/PBST, as previously described, and then washed 3 times for 5 minutes with PBST. Proliferation in whole larvae was measured using EDU staining, larvae were incubated in 10 µM 5-ethynyl-2'-deoxyuridine (EdU) dissolved in embryo medium for 6 hours. Larvae were euthanized and fixed in 4% PFA overnight at 4°C and stored in Methanol at -20℃ until staining. Click-iT EdU Imaging Kit (Life Technologies) were used for staining following manufacturer's instructions.

The following primary antibodies were used: rabbit anti-GAPDH (Abcam (Cambridge, CB2 0AX, UK), ab181602, 1:500), rabbit anti-Nitrotyrosine (Thermofisher, A-21285, USA, 1:200). Secondary antibodies were goat anti-rabbit Alexa Fluor 488 (#A-11008, 1:1,000) and Alexa Fluor 594 (#A-11012, 1:400).

· Confocal Microscopy Imaging- zWEDGI

All imaging was performed using a zWEDGI device as previously described (Huemer et al., 2017). Briefly, an anesthetized larva was loaded into a zWEDGI chamber for time-lapse imaging. The loading chamber was filled with 1% low melting point agarose (Sigma-Aldrich) in E3 to retain the larvae in the proper position. Additional E3 supplemented with 0.16 mg/ml Tricaine was added as needed to avoid dryness and provide required moisture to zebrafish larvae during imaging acquisition. All images were acquired on a spinning disk confocal microscope (CSU-X; Yokogawa) with a confocal scanhead on a Zeiss Observer Z.1 inverted microscope equipped with a Photometrics Evolve EMCCD camera, and an EC Plan Neofluar NA 0.3/10 x air objective, z-stacks, 5 µm optical sections and 512 x 512 resolution. For whole-larvae imaging, 7 x 1 tile images were taken and automatically stitched. For time-lapse movies of tailfin injury and neutrophil and chemotaxis, images were taken every 2 minutes up to 5 hours post-wounding. For photoconversion assay, images were taken every 15 minutes up to 6 hours post-photoconversion. For neutrophil migration parameters, images were taken every 2 minutes up to 4 hours. For tail wound experiments, the 10X objective was used. For neutrophil behavior in *spint1a* -/- larvae, the 40X objective was used.

· Automatic quantification of innate immune cells and cell-tracking on IMARIS

To quantify number of neutrophils of 3 dpf larvae were fixed and tailfin images were acquired as described previously. For quantification of the number of neutrophils in the wounded regions, acquired zebrafish tail images were reconstructed on IMARIS Bitplane software (Version 9.5/9.6) rendering mode and total number of neutrophils were automatically counted in the wound at various time points (0, 1-, 2-, 3-, 4- and 5 hours post wound) using IMARIS spots function. Spots were defined as particles with 5 µm and 10 µm of X/Y and Z diameter, respectively. Cells were automatically counted using the IMARIS spots function and defined as particles with 5 µm and 10 µm of X/Y and Z diameter, respectively. Cell tracking was performed in the field of view (FOV) and in the wounded area. To analyze the neutrophils interacting with the keratinocyte aggregates in the skin, a surface for each region/area representing each aggregate was created, then a mask for the neutrophil signals were generated setting to “zero” signal outside the surface. Mean neutrophil speed and displacement length were obtained by IMARIS cell tracking analysis. IMARIS volume rendering mode was used to obtained representative 3D reconstructions that were used for figures and supplemental movies.

· Western blot

Zebrafish embryos at 72 hpf were anesthetized in tricaine (200 µg/mL) dissolved in egg water, and the end of the fin fold was amputated with a scalpel. Tissues collected from around 40 embryos were pooled and then spun and resuspended in 200 µL of 10 mM Tris pH 7.4 + 1% SDS. Samples were then incubated at 95°C for 5 minutes with 1,400 rpm agitation, followed by maximum speed centrifugation for 5 minutes. Supernatants were frozen at −20°C until proceeding. BCA kit was employed to quantify protein using BSA as a standard. Fish lysates (20 µg) in SDS sample buffer were subjected to electrophoresis on a polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated for 1 hour 30 minutes with Tris Buffered Saline, with Tween 20, pH 8.0 (TTBS) containing 5% (w/v) skimmed dry milk powder and immunoblotted in the same buffer 16 hours at 4°C with the rabbit monoclonal antibody to human GAPDH (1/2500, Abcam, ab181602), the rabbit anti-Histone H3 (1/1000, Abcam, ab1791), and the rabbit polyclonal anti-beta actin (1:2500, Santa Cruz Biotechnology, sc-47778). The blot was then washed with TTBS and incubated for 1 hour at RT with secondary HRP-conjugated antibody diluted 2,500-fold in 5% (w/v) skimmed milk in TTBS. After repeated washes, the signal was detected with the enhanced chemiluminescence reagent and ChemiDoc XRS Bio-Rad.

· Analysis of gene expression

Total RNA was extracted from whole larvae or human cell pellets with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated with deoxyribonuclease I, amplification grade (RNA, 1 U/mg; Invitrogen). SuperScript IV RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with random primer from 1 µg of total RNA at 50°C for 50 min. Real-time PCR was performed with an ABIPRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and, last, 15 s at 95°C, 1 min 60°C, and 15 s at 95°C. For each mRNA, gene expression was normalized to the rps11 (zebrafish) or ACTB (human) content in each sample, using the Pfaffl method (Pfaffl, 2001). The primers used are shown in Table 3. In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples (Tyrkalska et al., 2022).

· GAPDH Activity assay

Zebrafish embryos at 3 dpf were anesthetized in tricaine (200 µg/mL) dissolved in egg water, and the end of the fin fold was amputated with a scalpel. Tissues collected from around 40 embryos were pooled and then spun and processed following the manufacturer's instructions (Abcam, Human GAPDH Activity Assay Kit II , ab284542). GAPDH activity was revealed by measuring absorbance at 450 nm using a plate reader POLARstar *Galaxy.* The absorbance at 450 nm correlates with the formation of an intermediate product between Glyceraldehyde 3-Phosphate (GAP) and biphosphoglycerate (BPG).

· Tissue dissection and single cell RNA-seq analysis

Around 100 zebrafish embryos at 3dpf of each group were anesthetized in tricaine (200 µg/mL) dissolved in egg water, and the fin fold was amputated at the end of the yolk sac extension using a scalpel. Tissues were pooled and resuspended in PBS with liberase 1:65/volume of PBS (Roche, #05401119001), and incubated at 28ºC for cell disaggregation. After processing, cell concentration was adjusted to 1000 cells/µL. The genetics and proteomics service of ACTI, in the Universidad de Murcia, prepared the scRNA-seq libraries using the 10x Genomics' Chromium Single Cell 3' V2 chemistry and processed using the 10x Cell Ranger pipeline.

The files generated with Cell Ranger were processed in R using Seurat package (version 4.3.0.1) (Satija, Farrell, Gennert, Schier, & Regev, 2015). Gene counts from Cell Ranger were imported using the function CreateSeuratObject, with a min.cells = 25 and min.genes =250. Low-quality cells were removed using the following thresholds: a minimum of 250 and maximum of 2500 genes, a maximum of 10% of mitochondrial gene UMIs (13 mitochondrial genes considered in

total) and a maximum of 15000 UMIs in total. JackStraw function (Chung & Storey, 2015) was used to predict and remove the cell doublets present in the single cell RNA-Seq. SCT transform normalization, which applies a regularized negative binomial regression and variance stabilization, was applied to the filtered dataset using the SCTransform function in Seurat (Hafemeister & Satija, 2019; Ratnayake et al., 2021). Data was normalized and scaled. Next, principal component analysis was carried out using the RunPCA function in Seurat, followed by a cell-clustering analysis with the functions FindNeighbours and FindClusters, both with a resolution = 0.5. UMAP and TSNE dimensional reduction was performed using the RunUMAP and RunTSNE function in Seurat (reduction.use = "pca", dims = 1:20). Cell clusters were identified and classified using the biomarkers obtained after applying the FindAllMarkers function, with a min.pct of 0.2 and using Wilcox statistic test. Despite the SCT transform, the assay data used was always the "RNA" assay. UMAP, feature plots and heatmaps were created using the DimPlot, FeaturePlot and DoHeatmap funcitions in Seurat. For the DotPlots and BarPlots generated, the package ggplot2 was used (Wu et al., 2021). To work individually with the different populations of neutrophils, basal keratinocytes and peridermal keratinocytes, the different clusters were isolated using the subset function, and were reclustering following the previous steps carried out with the global population.

· Gene Expression Omnibus (GEO) database

Human psoriasis (accession number: GSD4602) transcriptomic data were collected in the GEO database (https://www.ncbi.nlm.nih.gov/geo/). Gene expression plots were obtained using R Studio and the ggplot package (Wu et al., 2021).

· Immunohistochemistry in human skin samples

Skin biopsies from healthy donors (n = 4) and psoriasis patients (n = 4) were fixed in 4% PFA, embedded in Paraplast Plus, and sectioned at a thickness of 5 µm. After being dewaxed and rehydrated, the sections were incubated in 10 mM citrate buffer (pH 6) at 95°C for 30 minutes and then at RT for 20 minutes to retrieve the antigen. Afterward, steps to block endogenous peroxidase activity and nonspecific binding were performed. Then, sections were immunostained with rabbit monoclonal antibody to GAPDH (Abcam, ab181602, 1:500) followed by 1/100 dilution of biotinylated secondary antibody followed by ImmunoCruz goat anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific, #A-11012, 1:400). Finally, after DAB staining solution was added, sections were dehydrated, cleared, and mounted in Neo-Mount or directly stained with DAPI solution as indicated above. No staining was observed when primary antibody was omitted. Sections stained with DAB were finally examined under a Leica microscope equipped

with a digital camera Leica DFC 280, and the photographs were processed with Leica QWin Pro software. Sections stained with fluorescence were examined under a Stellaris confocal microscope with a 63× objective and further processed with Leica software.

· Human organotypic 3D models

Insert transwells (Merck, MCHT12H48) were seeded with 10^5 human foreskin keratinocytes (Ker-CT, ATCC CRL-4048) on the transwells in 300 µL CnT-PR medium (CellnTec) in a 12 well format. After 48 hours, cultures were switched to CnT-PR-3D medium (CELLnTEC, Bern 3014, Switzerland) for 24 hours and then cultured at the air–liquid interface for 17 days. From day 12 to 17 of the air–liquid interphase culture, the Th17 cytokines IL17A (30 ng/mL) and IL22 (30 ng/mL) were added (Smits et al., 2017). Pharmacological treatments were applied from day 14 to 17 and consisted of 10 µM CGP3466B. Culture medium was refreshed every 2 days. At day 17, the tissues were harvested for gene expression analysis.

· Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups with Gaussian data distribution (square root transformation were employed for percentage data). The differences between 2 samples were analyzed by the Student's *t* test. The contingency graphs were analyzed by the chi-squared (and Fisher exact) test.

Chapter 1: GAPDH nuclear translocation promotes chronic skin inflammation.

· Results

Oxidative stress, carried out by RONS, has been associated to different inflammatory diseases. In order to evaluate the levels of oxidative stress in the Spint1a-deficient line, a specific H_2O_2 fluorescence probe was used during 3 hours in wildtype and Spint1a deficient larvae of 72 hpf (**Figure 26A**). The results showed robust increased levels of ROS in Spint1a deficient larvae, localized mainly in the skin, where keratinocyte aggregates showed the highest levels of fluorescence intensity. By using the *Tg(lyz:dsRED2)*, which labels neutrophils, their recruitment to the skin sites with an increased ROS production was observed (**Figure 26B-C**).

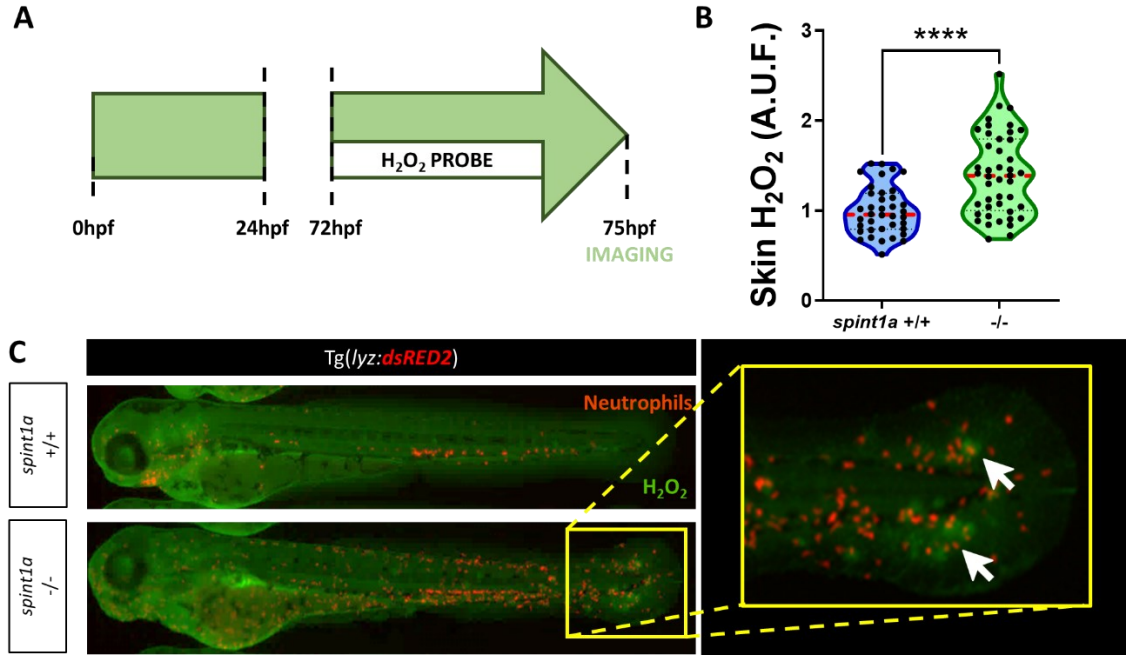

Figure 26. Skin oxidative stress and inflammation is altered in the Spint1a-deficient zebrafish line. (A) Wildtype and Spint1a deficient zebrafish embryos were treated for 3 hours with 50 µM of the H_2O_2 probe, acetyl-pentafluorobenzene sulphonyl fluorescein, by bath immersion, and then imaged at 75 hpf. **(B-C)** Quantification by fluorescence intensity of the ROS levels in the larval skin. Representative merged images showing neutrophils and H_2O_2 levels of wildtype and Spint1a-deficient larvae are shown. White arrows indicate keratinocyte aggregate regions, showing an increase in the H_2O_2 production. Each dot represents one individual, and the median for each group is also shown. *p*-Values were calculated using *t* test. ****$p \leq 0.0001$. A.U.F., arbitrary units of fluorescence.

Knowing the overproduction of ROS in the skin of Spint1a-deficient larvae, the next step consisted on elucidating if ROS were contributing to the aggravation of the chronic inflammatory phenotype observed. NOXs are one of the main producers of ROS in the cells (Forman & Zhang, 2021), so their inhibition was carried out in order to figure out whether they have a role in chronic skin inflammation. Manually dechorionated Spint1a deficient larvae were treated with apocynin, an inhibitor of NADPH oxidases (Savla, Laddha, & Kulkarni, 2021) during 48 h (**Figure 27A**). Spint1a-deficient larvae treated with apocynin showed strong decreased number of keratinocyte aggregates in the skin (**Figure 27B**), and reduced percentage of neutrophils

dispersed out of the caudal hematopoietic tissue (CHT) of the larvae compared to the control Spint1a-deficient larvae treated with DMSO (**Figure 27C-D**).

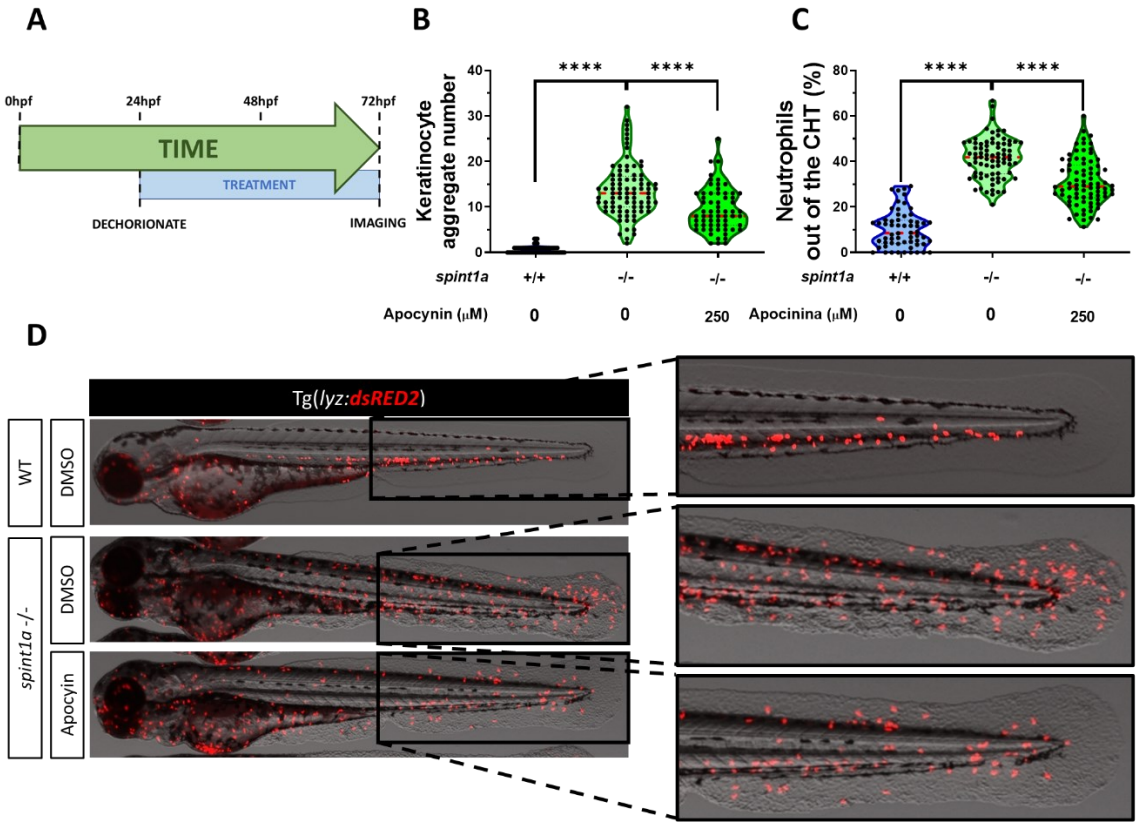

Figure 27. Apocynin treatment alleviates skin inflammation in Spint1a-deficient larvae. (A) Zebrafish embryos were manually dechorionated at 24 hpf, and treated with the inhibitor during 48 h. At 72 hpf, larvae were imaged and the phenotype was evaluated. **(B)** Quantification of the number of keratinocyte aggregates present in the skin of Spint1a-deficient larvae treated with 250 µM apocynin. **(C)** Quantification of the percentage of neutrophils dispersed in the skin. **(D)** Representative merged images showing neutrophils and the skin of wildtype, Spint1a deficient, and Spint1a deficient larvae treated with apocynin are shown. Yellow arrows indicate keratinocyte aggregates. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. ****p ≤ 0.0001. ANOVA, analysis of variance; WT, wildtype; DMSO, dimethyl sulfoxide; CHT, caudal hematopoietic tissue; hpf, hours post fertilization.

Once confirmed the role of NADPH oxidases in the development of chronic skin inflammation, the different enzymes of the family were subjected to an individual study to know their role in ROS production in a chronic inflammatory context. Nox1, Nox4, Nox5, Duox1 and Duox2 form the NOX protein family. As the role of Duox1 was previously pointed out in inflammation, playing a crucial role in neutrophil recruitment to inflamed skin (PMID: 24802997), the NOX enzymes were genetically inhibited in Spint1a-deficient larvae using CRISPR-Cas9 technology. Zebrafish egg were microinjected and they were imaged at 72 hpf (**Figure 28A**). Genetic inhibition of *nox1*, *nox4* and *nox5* leaded to an alleviation in the phenotype, observing a decrease in the number of keratinocyte aggregates (**Figure 28B-D**), and in the neutrophil dispersion (**Figure 28C-D**).

Deconvolution analysis carried out with the tool ICE, from Synthego ((Conant et al., 2022),https://ice.synthego.com/#/), determined the efficiency of the genetic inhibition of *nox1*, *nox4* and *nox5*, being, respectively, 52%, 40% and 25% (**Figure 29A-C**).

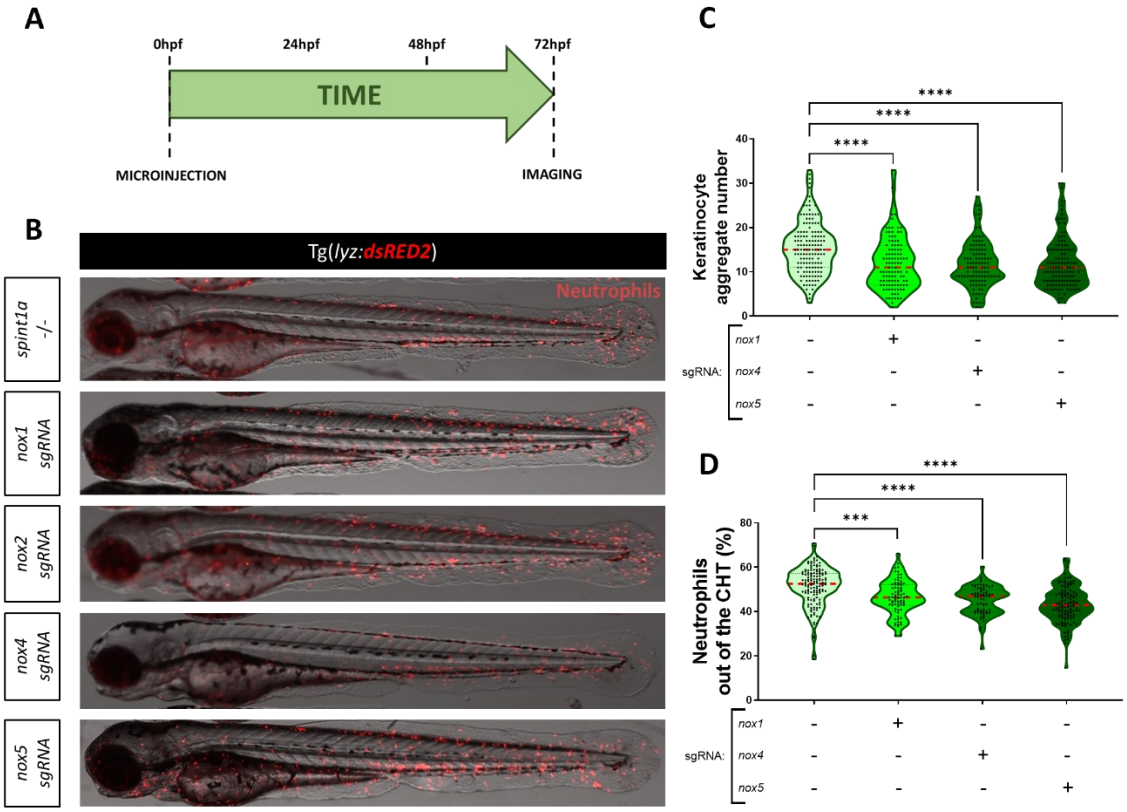

Figure 28. Genetic inhibition of *nox1*, *nox4* and *nox5* rescues the chronic inflammatory phenotype characteristic of the Spint1a-deficient line. (A) Zebrafish embryos were microinjected in one-cell stage of development with the crRNA of *nox1*, *nox4* and *nox5*. At 72 hpf the larvae were imaged and analyzed. **(B)** Representative merged images showing neutrophils and the skin of Spint1a-deficient larvae microinjected with a standard sgRNA and microinjected with the *nox* family crRNAs in the presence of recombinant Cas9. **(C)** Quantification of the number of aggregates of keratinocytes in the skin of the zebrafish larvae. **(D)** Quantification of the percentage of neutrophils infiltrated in the skin. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. ***p ≤ 0.001, ****p ≤ 0.0001. ANOVA, analysis of variance; crRNA, CRISPR RNA; *nox,* NADPH oxidase.

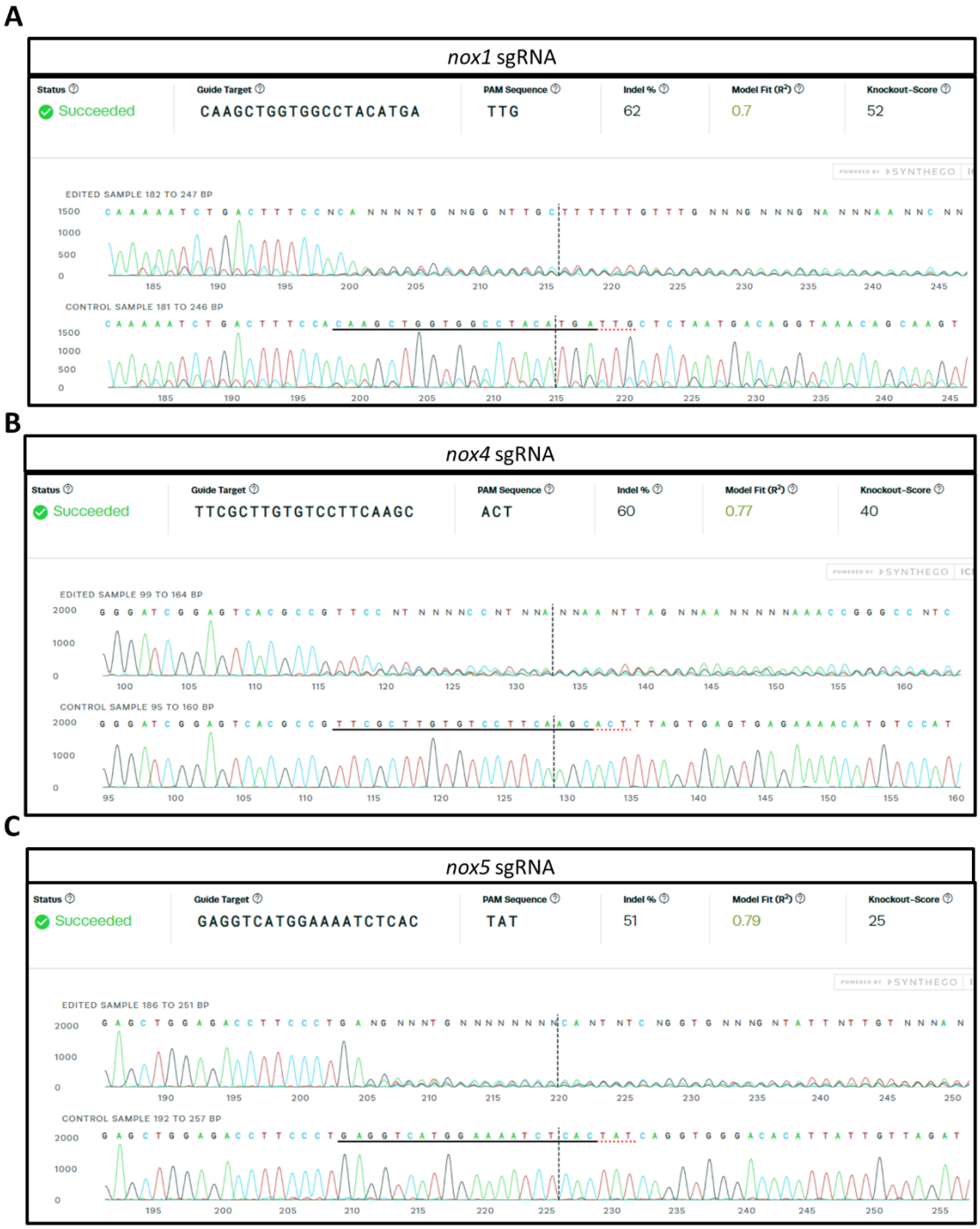

Figure 29. CRISPR editing efficiency after microinjection of the crRNAs of *nox1*, *nox4* and *nox5*. The open-source tool ICE from Synthego calculated the editing efficiency by comparing the sequence of larvae microinjected with the crRNA of the gene of interest versus control larvae microinjected with a standard crRNA in the presence of recombinant Cas9. (A) Larvae microinjected with *nox1* crRNA showed an indel % of 62 and a 52% of knockout score. (B) The indel % of the larvae microinjected with *nox4* crRNA was 60, with a 40% of knockout score. (C) Finally, larvae microinjected with *nox4* crRNA presented an indel % of 51 and a 25% of knockout score. CrRNA, CRISPR RNA; *nox*, NADPH oxidase.

Oxidative stress and ROS production are linked to nitrosative stress and the production of RNS and RONS, being peroxynitrite the most dangerous species reported (Al-Shehri, 2021; Forman & Zhang, 2021). Taking it into consideration, zebrafish wildtype and Spint1a-deficient larvae at 72

hpf were fixed and subjected to immunohistochemistry using an antibody which detects nitrosylation. Fluorescence intensity levels after imaging reflected the nitrosylation levels in the skin of the larvae. The results showed increased nitrosylated proteins levels in the skin of Spint1a-deficient larvae (**Figure 30A-B**). Moreover, the higher levels of nitrosylation were again predominantly observed in the keratinocyte aggregates (**Figure 30B**).

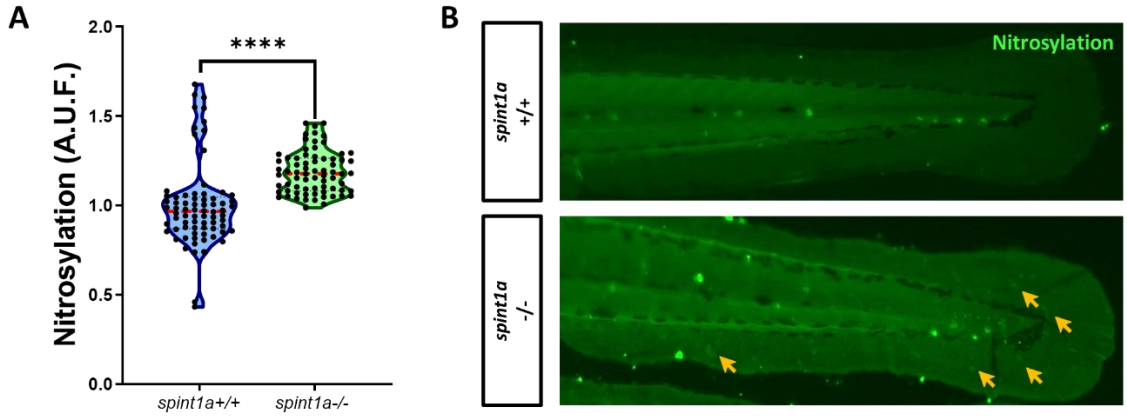

Figure 30. The skin of Spint1a-deficient larvae has high levels of nitrosative stress. An immunohistochemistry anti-nitrosylation was carried out in zebrafish *spint1a* +/+ and *spint1a* -/- larvae. **(A)** Nitrosylation levels measured by fluorescence intensity. **(B)** Representative images of nitrosylation in larval tail. Yellow arrows indicate the keratinocytes aggregates. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ****p ≤ 0.0001. A.U.F., Arbitrary Units of Fluorescence.

In order to check the impact of nitrosative stress in the phenotype, Spint1a-deficient larvae were treated for 48 h with linsidomine chlorhydrate (SIN-1), a nitric oxide donor that generates peroxynitrite (Wegner, Knispel, Meier, Klan, & Miller, 1995). SIN-1 treatment, as expected, triggered an aggravation of the phenotype, translated into an increase in the number of keratinocyte aggregates and in the percentage of neutrophils infiltrated in the skin (**Figure 31A-C**). As peroxynitrite is one of the most aggressive RNS, control *spint1a* +/+ larvae were treated with SIN-1 in the same conditions as Spint1a deficient larvae in order to evaluate its toxicity. However, no significant changes were observed between the percentage of neutrophils out of the CHT of Spint1a-deficient larvae treated with DMSO or SIN-1 (**Figure 31D**).

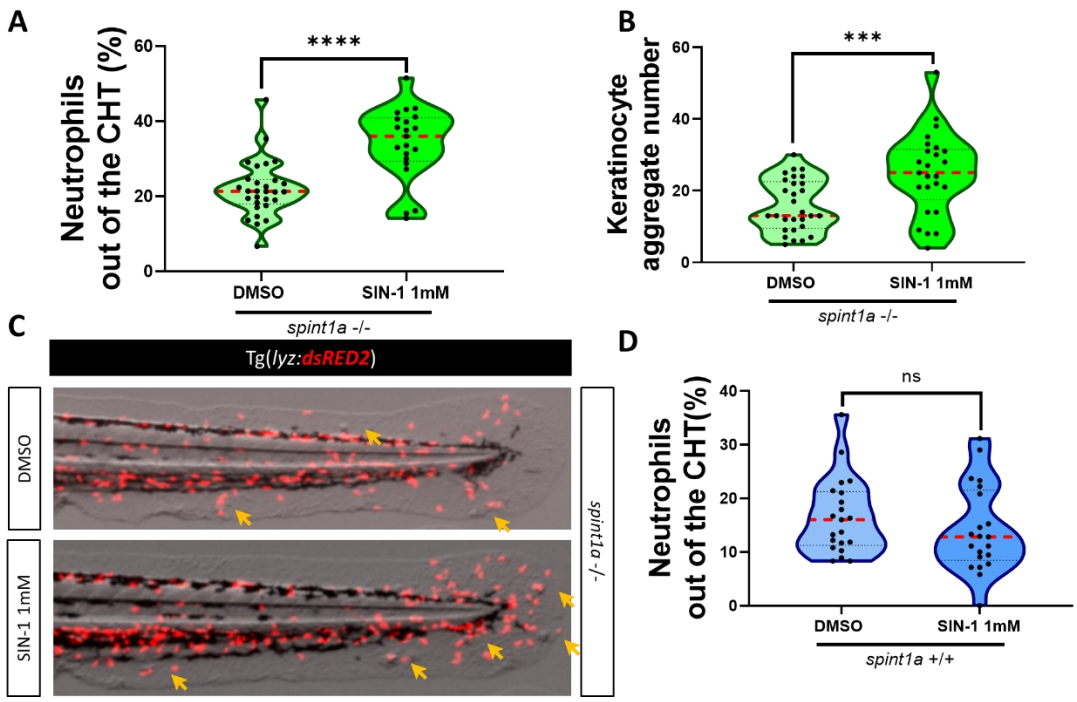

Figure 31. The nitric oxide donor SIN-1 aggravates skin inflammation of Spint1a-deficient larvae. Zebrafish embryos of *spint1a* -/- and *spint1a* +/+ lines were treated for 48 h with 1 mM SIN-1. **(A)** Percentage of neutrophils infiltrated in the skin, and **(B)** number of keratinocyte aggregates. **(C)** Representative merged images showing neutrophils and the skin of Spint1a-deficient larvae treated with DMSO and SIN-1. **(D)** Percentage of neutrophils infiltrated in the skin of control *spint1a* +/+ larvae treated with SIN-1. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ***p ≤ 0.001, ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; CHT, caudal hematopoietic tissue.

To determine if the results obtained correlate with what happens in psoriasis and atopic dermatitis patients, freely available transcriptomic data from healthy subjects and psoriasis and atopic dermatitis patients were analyzed. Among the studied genes, *NOX1-5*, *NOS1-3*, *COX1-2*, *XDH*, *DUOX*, *CAT*, *SOD1* and *SOD2* were analyzed. *DUOX1*, *XDH*, *NOS3* and *SOD2* showed increased transcript levels in both psoriasis and atopic dermatitis injured skin. Moreover, psoriasis patients' skin showed increased mRNA levels of *NOX1* and *NOS1-2*, while atopic dermatitis patients showed higher levels of *NOX4*. In addition, antioxidant genes like *SOD1* and *CAT* were downregulated in both conditions (**Figure 32A**). Data was represented in heatmaps for psoriaris patients (Fig. 32B) and psoriasis and atopic dermatitis patients (**Figure 32C**). *SOD2*, *XDH*, *CAT*, *SOD1*, *NOS2* and were the most significant differentially expressed genes, as shown in the heatmaps (**Figure 32B-C**).

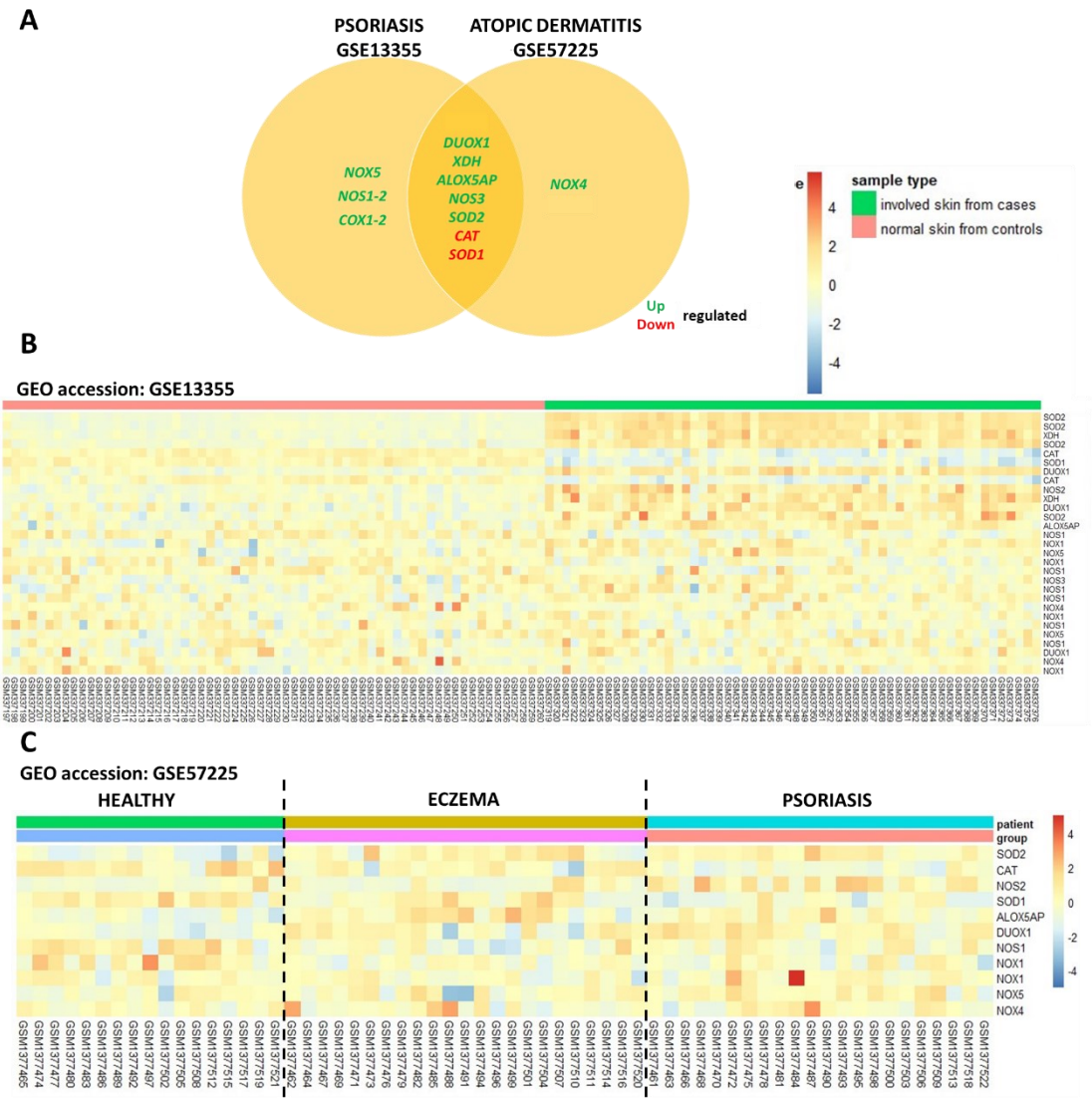

Figure 32. Differential expression profiles of genes encoding key oxidative and nitrosative enzymes in atopic dermatitis and psoriasis. Transcriptomic data from human psoriasis (GSE13355) and atopic dermatitis (GSE57225) samples from the Gene Expression Omnibus (GEO) database were analyzed. **(A)** Venn diagram showing common and specific gene transcript levels in each type of samples. **(B)** Heatmap showing analyzed gene transcript levels in psoriasis (GSE13355) samples versus control samples. **(C)** Heatmap showing analyzed transcript levels in atopic dermatitis (GSE57225) samples versus psoriasis and control samples. The heatmaps present duplicates or triplicates of the same gene because the experiment carried out was a microarray, containing different probes for the same gene.

The overproduction of ROS, RNS and, overall, peroxynitrite, leads to an enormous cell stress that, in many cases, triggers cell death by different pathways (Calabrese et al., 2007; Kubo et al., 2016; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021). With the aim of characterizing the Spint1a-deficient mutant model, a TUNEL assay was carried out in wild type and *spint1a* -/- larvae in order to know if there is an increase in cell death in a context of chronic skin inflammation. The experiment showed that, as hypothesized, the Spint1a-deficient mutant present more dead cells compared with the wild type larvae (**Figure 33A-B**). This result suggests

a correlation between the oxidative stress and the triggering of cell death in diseases like psoriasis or atopic dermatitis.

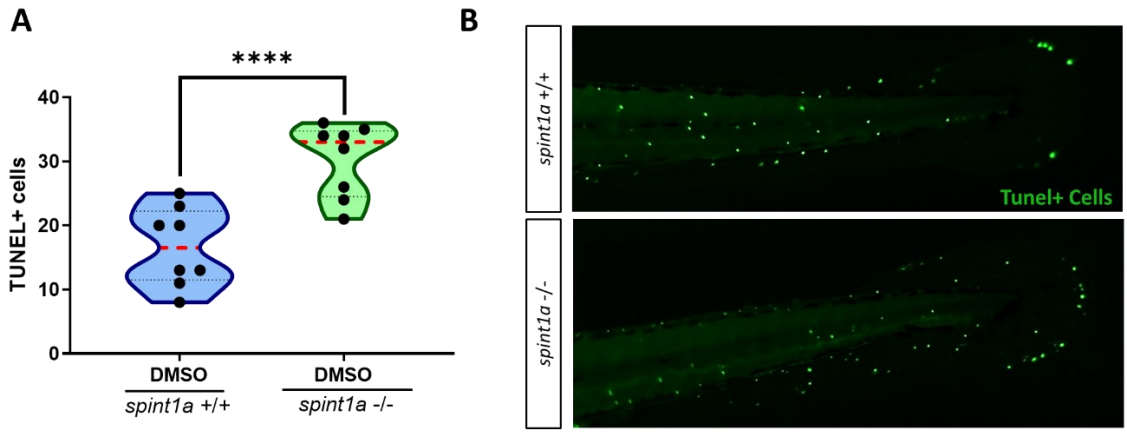

Figure 33. Spint1-deficient larvae have higher number of dead cells than wild type larvae. WT (*spint1a* +/+) and Spint1a deficient larvae were subjected to a TUNEL assay to determine cell death. **(A)** Number of dead cells of wild type and Spint1a-deficient larvae. **(B)** Representative images showing dead cells in *spint1a* +/+ and *spint1a* -/- larvae. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

We have recently reported that, in the Spint1a-deficient larvae, there was an alteration in the NAD^+ synthesis pathway that led to increased oxidative stress and *parthanatos* cell death (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021). The NAD^+-consuming enzyme PARP1 was pointed out as one of the main enzymes involved in cell death in this model, carrying out *parthanatos* after being overactivated because of the robust ROS-dependent DNA damage in keratinocytes (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021). Furthermore, it has been reported that the glycolytic enzyme GAPDH is nitrosylated and translocated to the nucleus during oxidative and nitrosative conditions, triggering a caspase-independent cell death process (Hara et al., 2005; Lavallard et al., 2009; N. Sen et al., 2008). Moreover, some papers have reported that GAPDH-PARP1 interaction in the nucleus activates PARP1 activity, a role for GAPDH in DNA repair (Kornberg et al., 2010; Kosova et al., 2017), and nuclear GAPDH-mediated NAMPT nuclear translocation, which increases NAD^+ levels in the nucleus (Grolla et al., 2020). All these results, together with the fact of GAPDH is another NAD^+-dependent enzyme, led us to think in GAPDH as a possible enzyme involved in the development and progression of chronic skin inflammation. We, therefore, performed an immunohistochemistry using an antibody detecting GAPDH in psoriasis skin biopsies. Two different samples were studied: skin biopsies of normal subjects with no skin diseases, and skin biopsies of lesional skin from psoriasis patients before any treatment. On the one hand, , we could observe that GAPDH was mainly expressed in the spinous stratum (SS) of the epidermis in

biopsies from healthy subjects, with some cells presenting high levels of the enzyme in their nucleus. There was no evidence of GAPDH expression in the dermis (D) (**Figure 34A**). On the other hand, the psoriasis biopsies showed a robust expression of GAPDH in the epidermis, observed along all the SS with the presence of many cells presenting the enzyme in their nucleus. Furthermore, the dermis of psoriasis patients showed GAPDH in the nucleus and in the cytosol of numerous cells, not only keratinocytes, due to the different cellular shapes observed (**Figure 34B).**

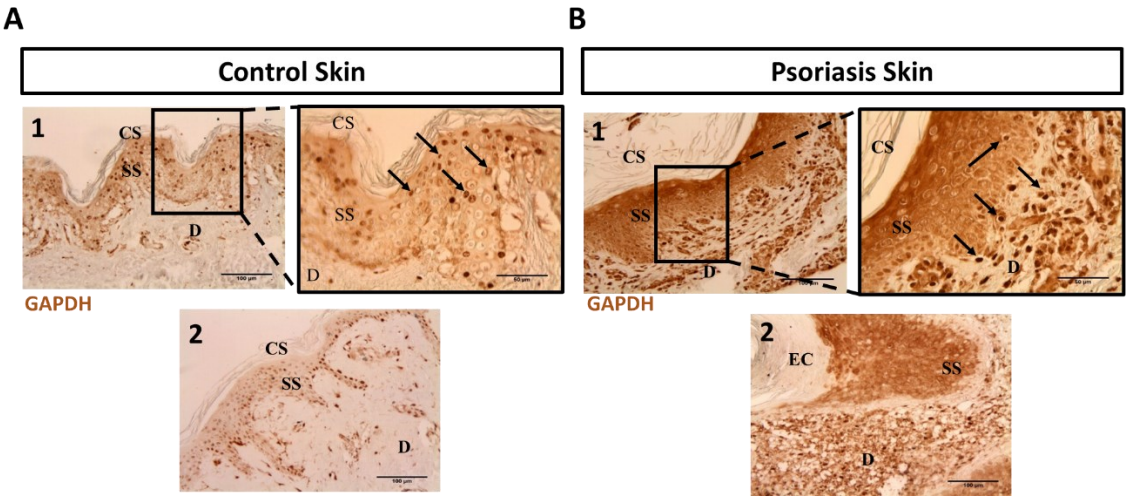

Figure 34. GAPDH shows increased levels and altered localization in the lesional skin of psoriasis patients. Representative images of skin biopsies of two healthy subjects (control skin, A) and two psoriasis patients (psoriasis skin, B) subjected to immunohistochemistry to detect GAPDH. The arrows indicated nuclear GAPDH. CS, corneum stratum; SS, spinous stratum; D, dermis; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase.

These results prompted us to elucidate the levels of Gapdh in wild type and Spint1a-deficient zebrafish (**Figure 35A**). Spint1a deficient larvae skin showed increased amount of Gapdh (**Figure 35B**) and they were reduced after the treatment with olaparib, a PARP1 inhibitor which has been found to decrease skin inflammation in Spint1a-deficient larvae and human organotypic skin model of psoriasis (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021) (**Figure 35C-D**). Furthermore, confocal immunohistochemistry images showed the presence of Gapdh in the nucleus of *spint1a* -/- skin cells, not observed in *spint1a* +/+ larvae cells (**Figure 35E**).

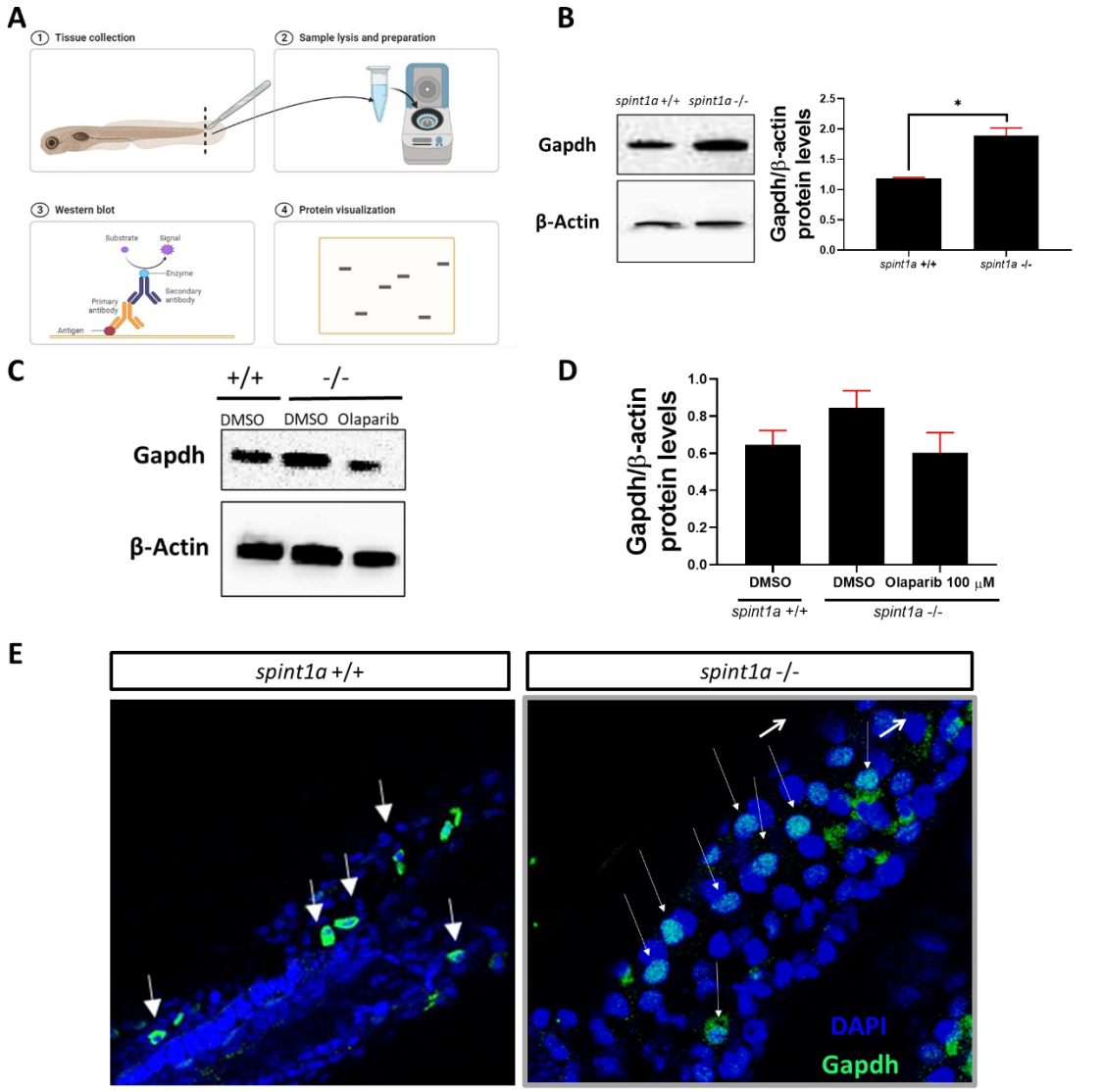

Figure 35. Gapdh protein levels are higher in the skin of Spint1a-deficient larvae. (A) The final part of the tail of 72 hpf larvae was cut in order to take only skin tissue. The samples were processed and used in a western blot to determine Gapdh levels. **(B, C, D)** Gapdh and β-actin protein amounts in tail skin of wild type and Spint1a-deficient larvae treated with either DMSO or olaparib. **(E)** Representative images of Gapdh immunohistochemistry of spint1a +/+ and spint1a -/- larvae at 72 hpf treated with DMSO. White arrows mark cell nucleus. Results are shown as the median ± SEM for each group. p-Values were calculated using t test and using 1-way ANOVA and Tukey multiple range test. *p≤0.05. DMSO, dimethyl sulfoxide; AUF, arbitrary units of fluorescence; Gapdh, Glyceraldehyde 3-Phosphate Dehydrogenase.

The high levels of GAPDH in psoriasis patients and Spint1a-deficient larvae, together with the nuclear localization of the enzyme in the biopsies, suggested a possible implication of GAPDH in the development and progression of the disease. Spint1a deficient larvae were treated for 24 h with CGP3466B, also known as omigapil, a specific inhibitor of GAPDH nuclear translocation (Godfrey et al., 2022; T. Sen et al., 2018) (**Figure 36A**). CGP3466B blocks the nitrosylation of the Cys^{152} of GAPDH, avoiding its binding to SIAH1 and the later nuclear translocation (**Figure 36B**). Mutant larvae treated with the inhibitor showed a strong reduction in the percentage of

neutrophils out of the CHT and decreased number of keratinocyte aggregates (**Figure 36C-E**). However, CGP3466B treatment did not inhibit Gapdh enzymatic activity and, in fact, Gapdh glycolytic activity increased in control larvae and no change was observed in Spint1a-deficient larvae (**Figure 36F-G**). Notably, wild type larvae showed slightly higher Gapdh enzymatic activity than Spint1a-deficient larvae, despite the latter had higher Gapdh protein amount.

With the aim of further confirm the impact of Gapdh nuclear translocation in the progression of skin inflammation, *siah1* gene was genetically inhibited using CRISPR/Cas9 technology in Spint1a-deficient larvae. As mentioned above, Gapdh should be unable to translocate to the nucleus in the absence of Siah1, despite being nitrosylated. As predicted, genetic inhibition of *siah1* ameliorated skin inflammation, as assayed as both the number of keratinocyte aggregates and the neutrophil dispersion of Spint1a-deficient larvae (**Figure 37A-C**).

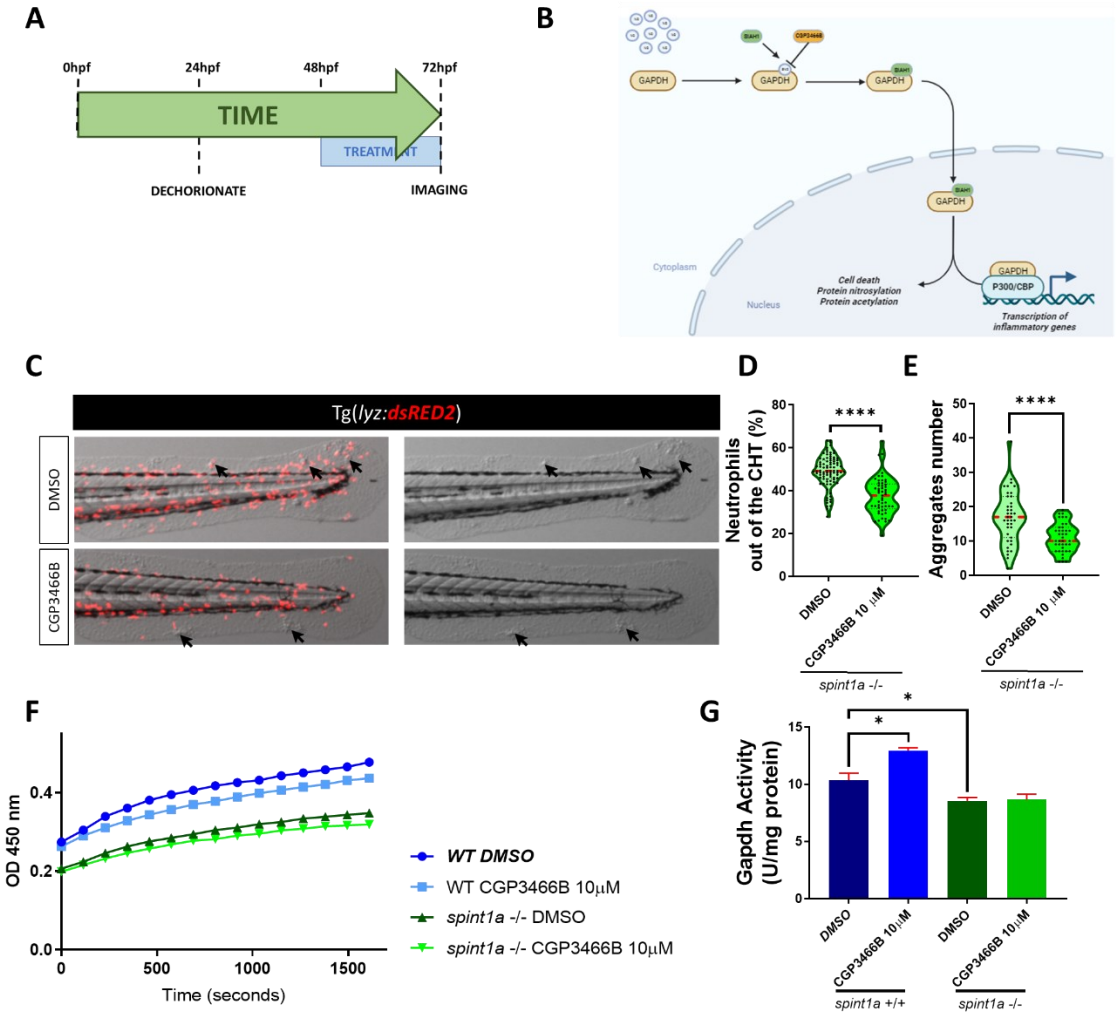

Figure 36. Gapdh nuclear translocation inhibition alleviates skin inflammation. (A) Zebrafish embryos were dechorinated at 24hpf, and treated with the inhibitor CGP3466B from 48 to 72 hpf. **(B)** CGP3466B inhibits GAPDH nitrosylation in the Cys^{152} residue of the protein, impairing its SIAH1-dependent nuclear translocation. **(C-D)** Representative merged images and quantitation of neutrophils dispersion **(D)** and keratinocyte aggregates **(E)** of Spint1a-deficient larvae treated with either DMSO or 10 µM CGP3466B.

Black arrows indicate keratinocyte aggregates. **(F, G)** Gapdh enzymatic activity assay showing optical density at 450 nm with time **(F)** and quantitation of Gapdh enzymatic activity **(G)** in wild type and Spnt1a-defcietn larvae treated with either DMSO or CGP3466B. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test and using 1-way ANOVA and Tukey multiple range test. *p≤0.05; ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; OD, Optical Density; U, Unit of enzymatic activity.

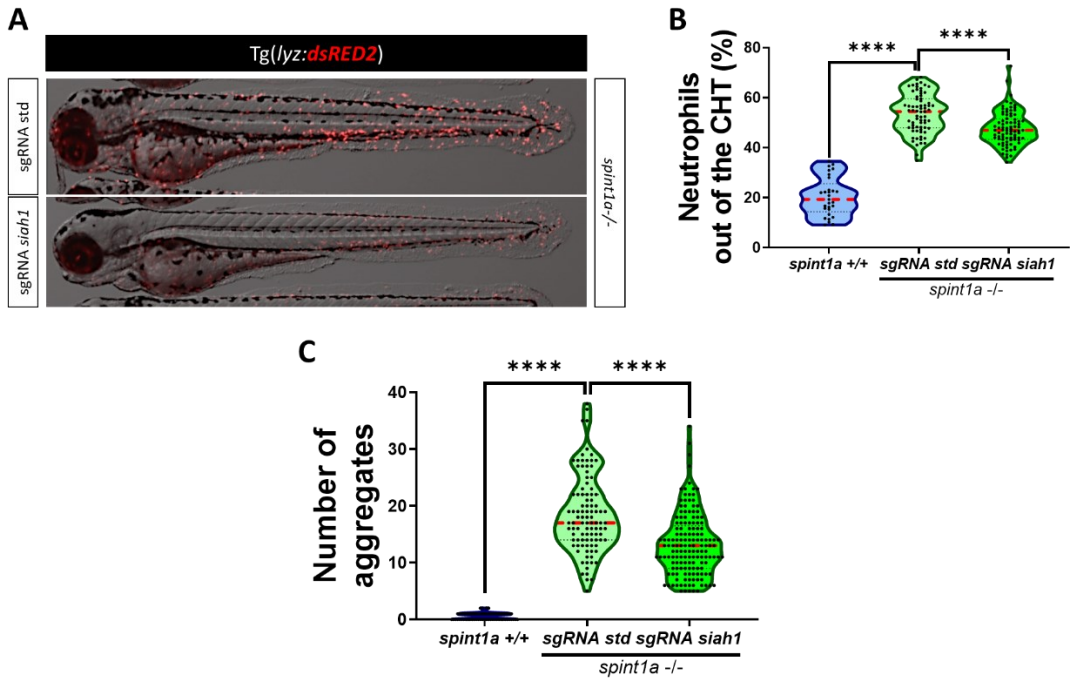

Figure 37. Genetic inhibition of *siah1* ameliorates chronic skin inflammation. (A) Representative merged images showing neutrophils and the skin of Spint1a deficient larvae microinjected with a standard (std) crRNA and with *siah1* crRNA in the presence of recombinant Cas9. **(B, C)** Percentage of dispersed neutrophils **(B)** and number of keratinocyte aggregates **(C)** of wild type and Siah1-deficient larvae. *p≤0.05; ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; crRNA, CRIPSR RNA.

We next analyzed Gapdh localization by whole-mount immunohistochemistry in Spint1-deficient larvae treated with either DMSO or CGP3466B and the results revealed that the inhibitor reduced Gapdh protein amount and its nuclear localization (**Figure 38A-B**). Furthermore, the TUNEL assay showed that treatment with CGP3466B resulted in reduced number of dead cells in Spint1a-deficient larvae, with values much more similar to their *spint1a* +/+ siblings (**Figure 38C-D**). Spint1a deficient zebrafish larvae were shown to present an increased number of proliferative cells in the skin, reduced after treatment with drugs which decrease chronic inflammation (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021). EdU assay revealed also a decrease in the number of proliferative cells observed in the skin of Spint1a deficient larvae after treatment with the inhibitor of the nuclear translocation of Gapdh (**Figure 38E-F**).

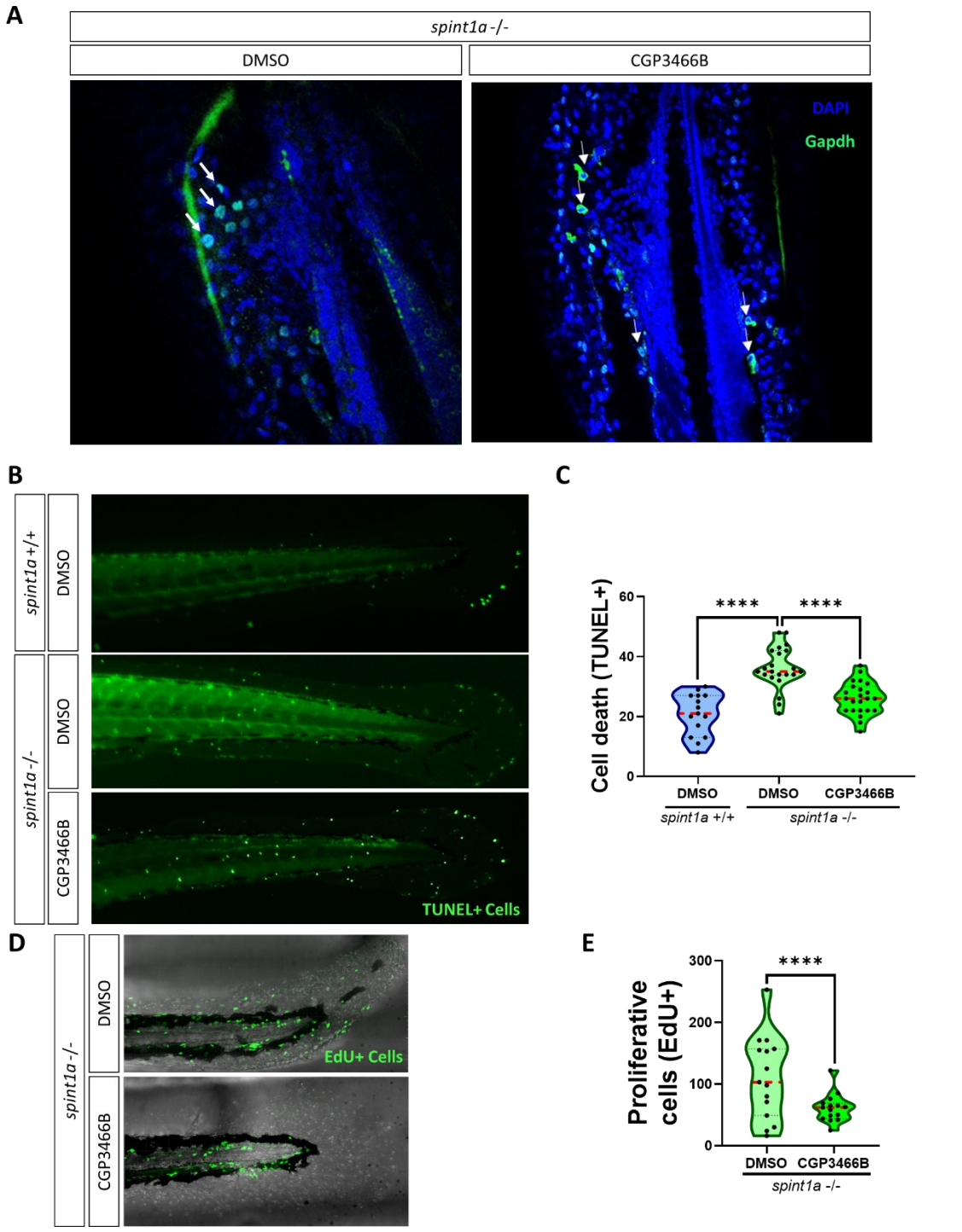

Figure 38. Gapdh nuclear translocation inhibition reduces keratinocyte death and hyperproliferation of Spint1a-deficient larvae. (A) Representative images of Gapdh immunohistochemistry of *spint1a* -/- larvae at 72 hpf treated with DMSO and 10 µM CGP3466B. White arrows mark cell nucleus. **(B)** Quantitation of Gapdh levels and localization in the skin of Spint1a-deficient larvae. **(C)** Representative images of TUNEL assay of *spint1a* +/+ and *spint1a* -/- larvae at 72 hpf treated with DMSO and 10 µM CGP3466B . **(D)** Quantitation of the number of cell death (TUNEL^{+}) observed in Spint1a-deficient and wild type larvae. **(E)** Representative images of EdU^{+} cells of *spint1a* -/- larvae treated with DMSO and 10 µM CGP3466B . **(F)** Quantitation of the number of proliferative cells (EdU^{+}) observed at 72 hpf. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test and using 1-way ANOVA and Tukey multiple range test. ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; TUNEL, Terminal

deoxynucleotidyl transferase dUTP nick-end labeling; EdU, 5-ethynyl-20- deoxyuridine assay; AUF, Arbitrary Units of Fluorescence.

We next wondered whether a similar mechanism operates in human skin. To do so, organotypic 3D human psoriasis models were used to determine the impact of GAPDH nuclear translocation (**Figure 39A**). The 3D psoriatic epidermis showed increased expression of inflammation markers associated to psoriasis, such as *NAMPT, DEFB4* and *S100A8,* after stimulation with cytokines IL-17 and IL-22 (**Figure 39B-D**). Interestingly, after treatment with CGP3466B, the expression of the psoriasis markers was strongly reduced (**Figure 39B-D**), suggesting that GAPDH nuclear translocation is also important in human skin inflammation.

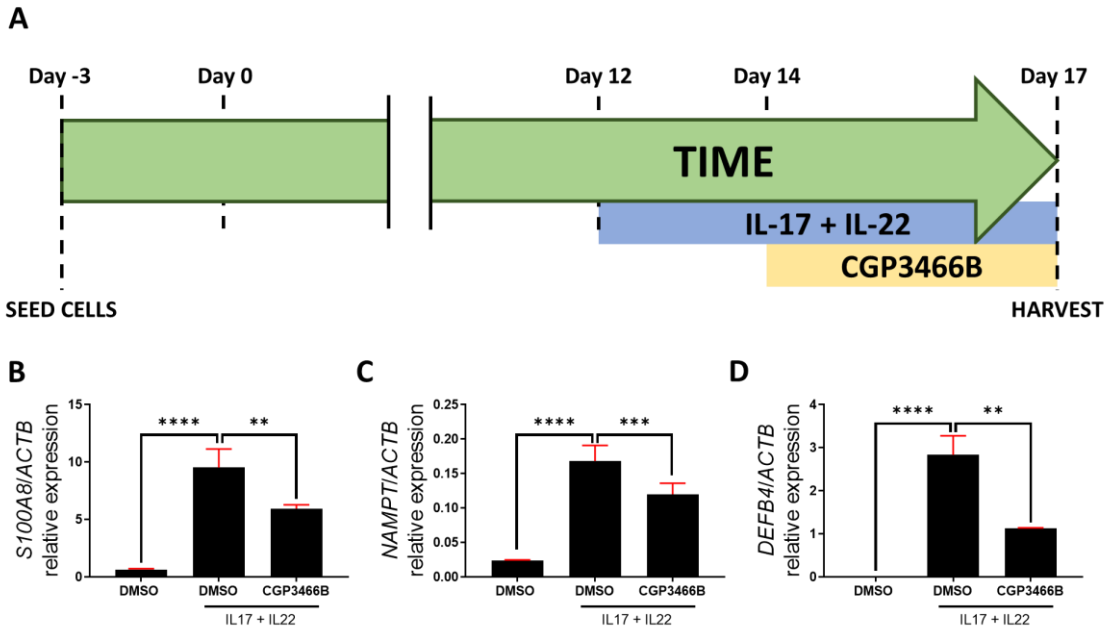

Figure 39. GAPDH nuclear translocation inhibition reduces pathology-associated gene expression in human organotypic 3D skin models of psoriasis. (A) After seeding, human keratinocytes were growth during 3 days before the beginning of the experiment. Keratinocytes were maintained until day 12, when IL-17 and IL-22 were added to the cell media, inducing psoriasis-like inflammation. At day 14, and after 2 days with the inflammatory stimulus, cultures were treated with 100 µM CGP3466B until day 17, when 3D organoids were harvested and processed for RNA extraction. The gene expression levels of psoriasis markers S100A8 **(B)**, NAMPT **(C)**, and DEFB4 **(D)** were analyzed by RT-qPCR. The mean ± SD for each group is shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

· Discussion

In this chapter, new insights into the pathways involved in chronic skin inflammation in zebrafish and human have been described. NADPH oxidase family have been pointed out as an important factor in chronic skin inflammation. Genetic inhibition of *nox1*, *nox4* and *nox5* and pharmacologic inhibition of all NAPDH oxidases with apocynin ameliorate larval skin inflammation, assayed as reduced number of neutrophils infiltrated in the skin and keratinocyte aggregates. NOX2 is an important enzyme for ROS production in neutrophils, provoking the removal of invading pathogens (Amara et al., 2021), although it is also expressed in keratinocyte after injury (Korkmaz et al., 2020). Meanwhile, NOX1 is expressed in intestinal and skin epithelia (N. Y. Hsu et al., 2023; Meyskens & Liu-Smith, 2017), NOX4 in skin fibroblast (Noh et al., 2017), and NOX5 also in keratinocytes (H. Choi et al., 2010). However, additional experiments would be required to determine the cell source of ROS contributing to skin inflammation.

Apart from oxidative stress, Spint1a-deficient larvae also showed higher nitrosative stress than control *spint1a* +/+ larvae and treatment with the peroxynitrite-donor SIN-1 aggravated skin inflammation of Spint1a-deficient larvae. However, the absence of negative effect of SIN-1 in control larvae suggest that, on its own, RNS are not able to trigger inflammation, but they could act as a positive feedback loop factor that aggravate chronic skin inflammation. Nitrosative stress have been mainly described in the context of neuronal inflammation and cell death in diseases like heart stroke (Chamorro, Dirnagl, Urra, & Planas, 2016), although it has been also reported in plaque psoriasis patients and mice models of psoriasis (Sangaraju, Alavala, Nalban, Jerald, & Sistla, 2021; Skutnik-Radziszewska et al., 2020; Sunkari, Thatikonda, Pooladanda, Challa, & Godugu, 2019). In order to know more about the mechanisms involving nitrosative stress and chronic skin inflammation, it would be interesting to ascertain the effects of the different nitric oxide synthases (NOSs) by using loss-of-function experiments. Although the results obtained with the zebrafish model are not conclusive, patient gene expression data also support a pathogenic role for ROS and RNS in psoriasis and atopic dermatitis.

Spint1a-deficient larvae also showed increased keratinocyte cell death. These results are in agreement with previous studies that reported that keratinocytes of this model suffered a type of cell death different from apoptosis, and later on it was identified as PARP1-dependent *parthanatos* cell death (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021; Mathias et al., 2007). Apical cell extrusion and *anoikis* has also been recently reported to occur in Spint1a-deficient larvae (Armistead et al., 2020). Nitrosative and oxidative stress have been previously linked to cell death by different pathways, such as apoptosis, necrosis, pyroptosis,

necroptosis and *parthanatos* (Kajarabille & Latunde-Dada, 2019; Varga et al., 2015), and different types of cell death have been found in chronic skin inflammation, including necroptosis, ferroptosis and *parthanatos* (de Reuver et al., 2022; Jiao et al., 2020; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021; Shou, Yang, Yang, & Xu, 2021), both nitrosative/oxidate stress and cell death are both attractive for the development of new treatments for psoriasis and atopic dermatitis.

Taking into account the increased oxidative and nitrosative stress and cell death, and the previously described alteration and involvement of NAD^+ metabolism in the skin inflammation developed by the Spint1a-deficient larval model (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021; Mercurio et al., 2021; Virag et al., 2002), we wanted to identify others enzymes that consumes NAD^+, whose activity is regulated by oxidative and nitrosative stress, and that could be involved in cell death, and, therefore, the moonlighting enzyme GAPDH was considered to fulfill all these requirements. Immunohistochemical and western blot analysis of Spint1a-deficient larvae and human skin biopsies revealed the overproduction of GAPDH in the inflamed skin. Strikingly, although control subjects showed some keratinocytes in the epidermis with high levels of GAPDH in their nucleus, which may suggest a physiological role of GAPDH in the removal of keratinocytes, GAPDH was hardly detected in healthy dermis. In sharp contrast, GAPDH was robustly accumulated in both the nucleus and cytosol of epidermal keratinocytes of psoriasis lesional skin and in the nucleus of several cells of the dermis. It is tempting to speculate that these cells are infiltrating immune cells that translocated GAPDH upon hyperactivation. Although further experiments are required to confirm this motivating and ambitious hypothesis, our results suggest that the translocation of GAPDH to the nucleus may be used as a diagnosis biomarker in psoriasis.

The relevance of nuclear GAPDH in inflamed skin was demonstrated for the first time using the Spint1a-deficient larval model where the GAPDH nuclear translocation inhibitor CGP3466B not only reduced the number and percentage of neutrophils infiltrated in the skin but also the number of keratinocyte aggregates, hyperproliferation and cell death. Importantly, CGP3466B did not affect Gapdh enzymatic activity, demonstrating that Gapdh nuclear translocation rather than its enzymatic activity is promoting skin inflammation. Furthermore, Gapdh activity was found to be lower in Spint1a-deficient larvae than in their wild type siblings, probably reflecting the inhibition of Gapdh enzymatic activity by oxidative and nitrosative stress and its subsequent nuclear translocation. These results were further confirmed by the genetic inhibition of *siah1* in the zebrafish model and by pharmacological inhibition of GAPDH nuclear translocation with CGP3466B in human organotypic 3D psoriasis models (Arroyo et al., 2023; Fatas-Lalana et al.,

2022; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021; Smits et al., 2017). Collectively, these results pointed to GAPDH nuclear translocation as an important driver of chronic skin inflammation and as a promising target for therapeutical intervention in chronic skin inflammatory disorders.

Finally, we also observed that inhibition of Gapdh nuclear translocation with CGP3466B led to decreased Gapdh nuclear protein levels in Spint1a-deficient larvae. Further experiments are required to understand whether inhibition of nuclear Gapdh resulted in its degradation, the mechanism involved and its relevance on its canonical function.

Chapter 2: The paradox between two different Gapdh enzymes: nuclear translocation of Gapdh, but not Gapdhs, regulates skin chronic inflammation in Spint1a-deficient larvae

· Results

In order to understand the different events that are taking place in the Spint1a-deficient mutant model of chronic skin inflammation, single cell RNA-seq (scRNA-seq) experiments were carried out in pooled tails of control *spint1a* +/+ and mutant *spint1a* -/-. The analysis revealed a total of 34 different clusters that were grouped in 17 different mayor clusters according to the cellular type and origin (**Figure 40A**). The clusters were classified using specific cell markers as endothelial cells, neutral crest, notochord, neuron precursors, mesoderm, otic cells, proliferative cells, glia cells, motor neurons, fibroblasts, erythrocytes, peridermal keratinocytes, basal keratinocytes, hematopoietic stem and precursor cells, macrophages, neutrophils and muscle cells (**Figure 40B**).

We focused our attention on the two different populations of keratinocytes: peridermal keratinocytes, which were completely differentiated and expressed markers such as *krt17* (Depianto et al., 2010), and basal keratinocytes, which expressed *krt91* or *tp63* (Truong et al., 2007) (**Figure 40B**).

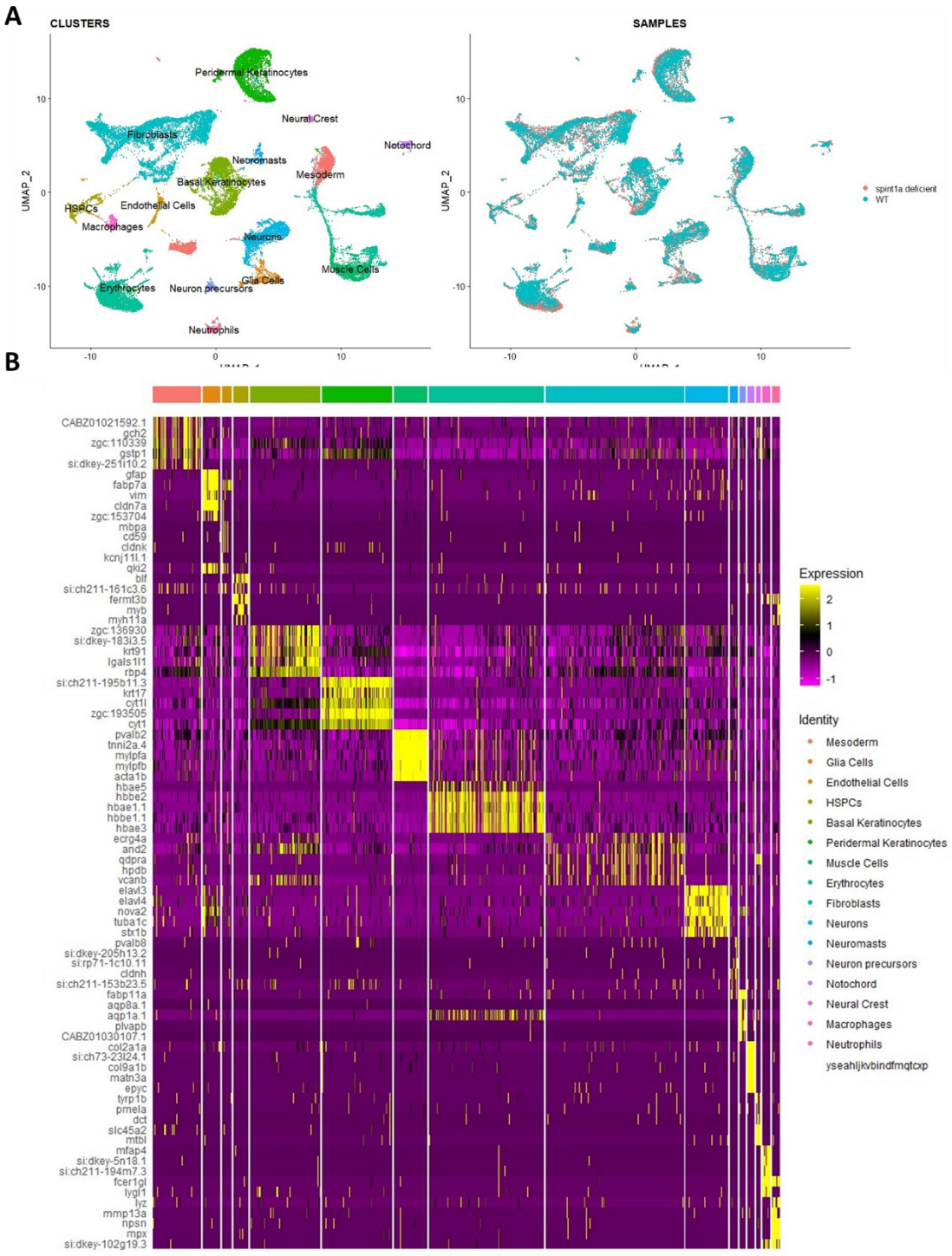

Figure 40. Single cell RNA-seq of tails of wild type and Spint1a-deficient larvae reveals 17 different clusters. (A) UMAP representation of the different cells analyzed by scRNA-seq. Left, cluster annotation can be observed, with 17 different clusters annotated in distinct colors. Right, cells are classified depending of the sample of origin: wild type (WT) or Spint1a-deficient larvae. **(B)** Heatmap showing the 5 most differentially expressed genes of each annotated cluster. The list was determined using Wilcoxon Rank Sum test to identify differentially expressed genes between groups. UMAP, Uniform Manifold Approximation.

Peridermal and basal keratinocytes were individually isolated and gene expression profile of Spint1a-deficient and wild type keratinocytes analyzed. Firstly, peridermal keratinocytes were selected by expression of the markers showed in Fig. 40B and processed (**Figure 41A**). Differential gene expression analysis comparing wild type and Spint1a-deficient peridermal keratinocytes revealed 242 differentially expressed genes. Among them, genes like annexin A1b (*anxa1b*) and glutathione S-transferase, alpha tandem duplicate 1 (*gsta.1*) were the most upregulated genes in the Spint1a-deficient peridermal keratinocytes, meanwhile transcobalamin beta b (*tcnnb*) and the non-annotated genes *si:dkey-248g15.3* and *si:dkeyp-51b9.3*, whose Blastp alignment of their amino-acid sequences revealed homologies with human Solute Carrier 39A7 (SLC39A7) and Dynein light chain (DYNLL1), respectively, were the most downregulated (**Figure 41B-C**). Gene enrichment pathway analysis showed several altered signaling pathways after comparing the peridermal keratinocytes of both samples, including biosynthesis and metabolism of fatty acids, biosynthesis of prostaglandins, prostanoids and eicosanoids, and keratinocyte differentiation (**Figure 41D**).

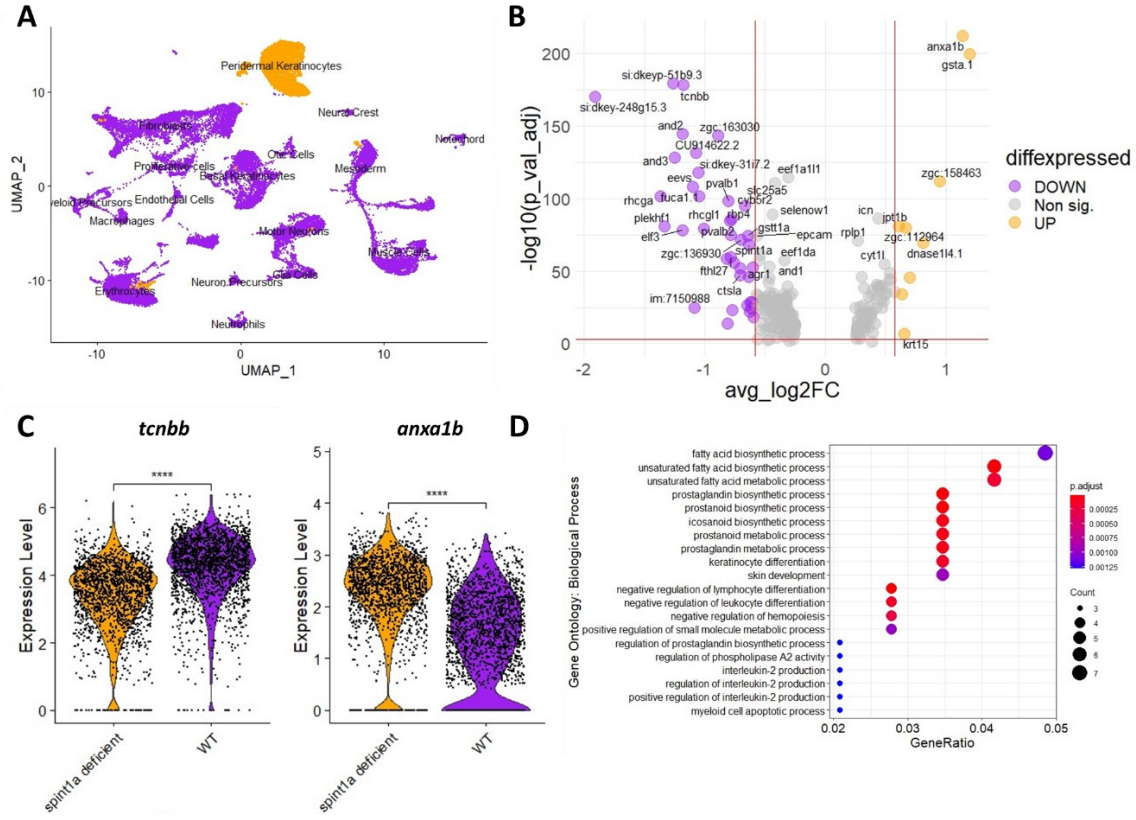

Figure 41. Gene expression profiles of peridermal keratinocytes of Spint1a-deficient and wild type larvae. (A) UMAP representation showing peridermal keratinocytes, highlighted in orange, that were selected among all the clusters obtained. **(B)** Volcano Plot showing 242 genes differentially expressed, highlighting those genes with a logFC ≥ 0.322 and ≤ -0.322, and a log10(p-value) ≥ 0.05. In yellow, genes upregulated in Spint1a-deficient peridermal keratinocytes are shown, and in purple, genes downregulated. **(C)** Violin Plot representation of the gene expression levels of two of the most

differentially expressed genes, *tcnbb* and *anxa1b*. Each point represents the gene transcript level of each cell analyzed. p-Values were calculated using Wilcoxon Rank Sum test. ****p ≤ 0.0001. **(D)** Dot plot showing the most altered pathways annotated by Gene Ontology: Biological Process in the peridermal keratinocytes of *spint1a* -/- larvae compared to spint1a +/+ larvae. Size of the dot and color depends on the number of genes differentially expressed included in the pathway and its p-adjusted-value, respectively. Bonferroni algorithm was used for multiple testing correction. UMAP, Uniform Manifold Approximation and Projection; FC, Fold Change.

Basal keratinocytes were selected based on the expression of markers like *krt91* and *cldni* (**Figure 40B**), and the cluster was analyzed as indicated above for peridermal keratinocytes (**Figure 42A**). In this case, the differential expression analysis showed 365 differentially expressed genes. *spint1a* and parvalbumin 2 (*pvalb2*) were the most downregulated in the basal keratinocytes of Spint1a-deficient larvae, and S100 calcium binding protein W (*s100w*) or the non-annotated gene *zgc:158463*, whose Blastp protein alignment showed homology with human cathepsin X (CTSZ) (**Figure 42B-C**), ones the most upregulated. The number of enriched pathways altered in basal keratinocytes of Spint1a-deficient model in comparison with *spint1a* +/+ were related to tissue development and organization (**Figure 42D**).

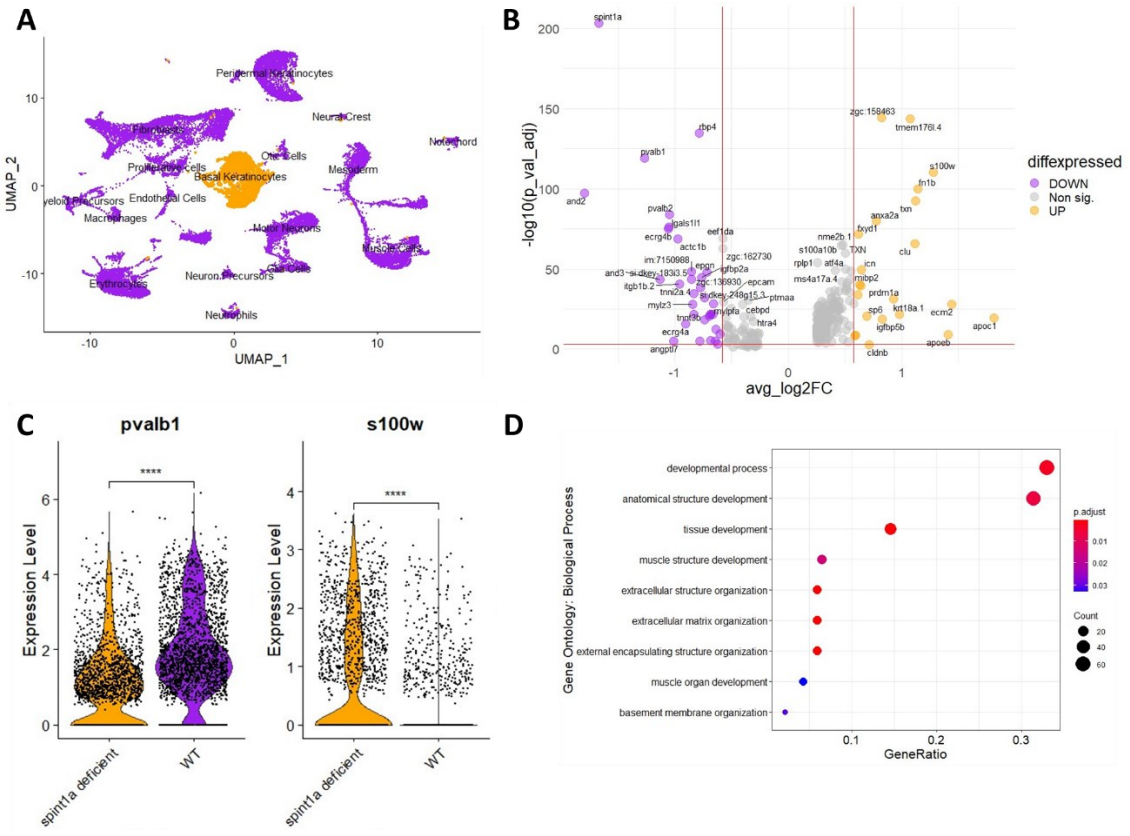

Figure 42. Gene expression profile of basal keratinocytes of Spint1a-deficient and wild type larvae. (A) UMAP representation showing basal keratinocytes, highlighted in orange, that were selected among all the clusters obtained. **(B)** Volcano Plot showing 365 genes differentially expressed, highlighting those genes with a logFC ≥ 0.322 and ≤ -0.322, and a log10(p-value) ≥ 0.05. In yellow, genes upregulated in Spint1a deficient basal keratinocytes are shown, and in purple, genes downregulated. **(C)** Violin Plot representation of the gene expression levels of two differentially expressed genes, *pvalb1* and *s100w*. Each point represents the gene expression level in a different cell analyzed. p-Values were calculated using

Wilcoxon Rank Sum test. ****p ≤ 0.0001. **(D)** Dot plot showing the most altered pathways annotated by Gene Ontology: Biological Process in the basal keratinocytes of spint1a -/- compared to spint1a +/+ larvae. Size of the dot and color depends on the number of genes differentially expressed included in the pathway and its p-adjusted-value, respectively. Bonferroni algorithm was used for multiple testing correction. UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

Data obtained from the analysis of peridermal and basal keratinocytes in Spint1a-deficient larvae did not provide obvious clues on the mechanisms of Gapdh nuclear translocation. For this reason, in the next step, the expression of the two genes encoding Gapdh, namely *gapdh* and *gapdhs*, was analyzed in the scRNA-seq data. Strikingly, *gapdhs* showed a general expression in almost all the cell clusters, with the lowest levels observed in muscle cells (**Figure 43A and C**), while *gapdh* expression was only observed in two specific clusters: neutrophils and muscle cells (**Figure 43A and B**).

Due to the surprising specific expression of canonical *gapdh* in muscle cells and neutrophils, and the different expression pattern compared to *gapdhs*, the next objective was to check if the specific inhibition of *gapdh* and *gapdhs*, individually and combined, showed the same effect in the Spint1a-deficient phenotype as the treatment with the inhibitor CGP3466B. With this in mind, CRISPR-Cas9 technology was used to inhibit *gapdh* and *gapdhs*. The genetic inhibition of *gapdh* with three different crRNAs (called AA, AB and AD) individually showed a decrease in the number of keratinocyte aggregates and neutrophil dispersion out of the CHT in 3 dpf Spint1a-deficient larvae (**Figure 44A-D**). For the next experiments, crRNA *gapdh* AA was used because the best results were obtained with it. Surprisingly, genetic inhibition of *gapdhs* did not reduce the number of keratinocyte aggregates or neutrophil dispersion. Moreover, the genetic inhibition of both *gapdh* and *gapdhs* gave similar results to the specific genetic inhibition of *gapdh* (**Figure 44D-F**).

In order to check the efficiency of the crRNA microinjection and in view of the impossibility of amplifying the target region, RT-qPCR analysis of complete larvae was performed. The results showed decreased transcript levels of *gapdh* and increased levels of *gapdhs* after the editing of *gapdh*, meanwhile the genetic inhibition of *gapdhs* resulted in decreased expression of both *gapdh* and *gapdhs* mRNA levels. Moreover, microinjection of the crRNA of *gapdh* and *gapdhs* together, led to decreased both mRNA levels (**Figure 45A-B**). Western blot analysis further confirmed these results, since a robust Gapdh protein amount reduction after the genetic inhibition of *gapdhs* alone or together with *gapdh* was observed. However, individual inhibition of *gapdh* alone did not significantly affect Gapdh protein amount (**Figure 45C**). Similar results were observed by immunohistochemistry. Thus, measurement of the fluorescence intensity in the skin of Spint1a-deficient larvae showed decreased Gapdh protein levels in the skin of the

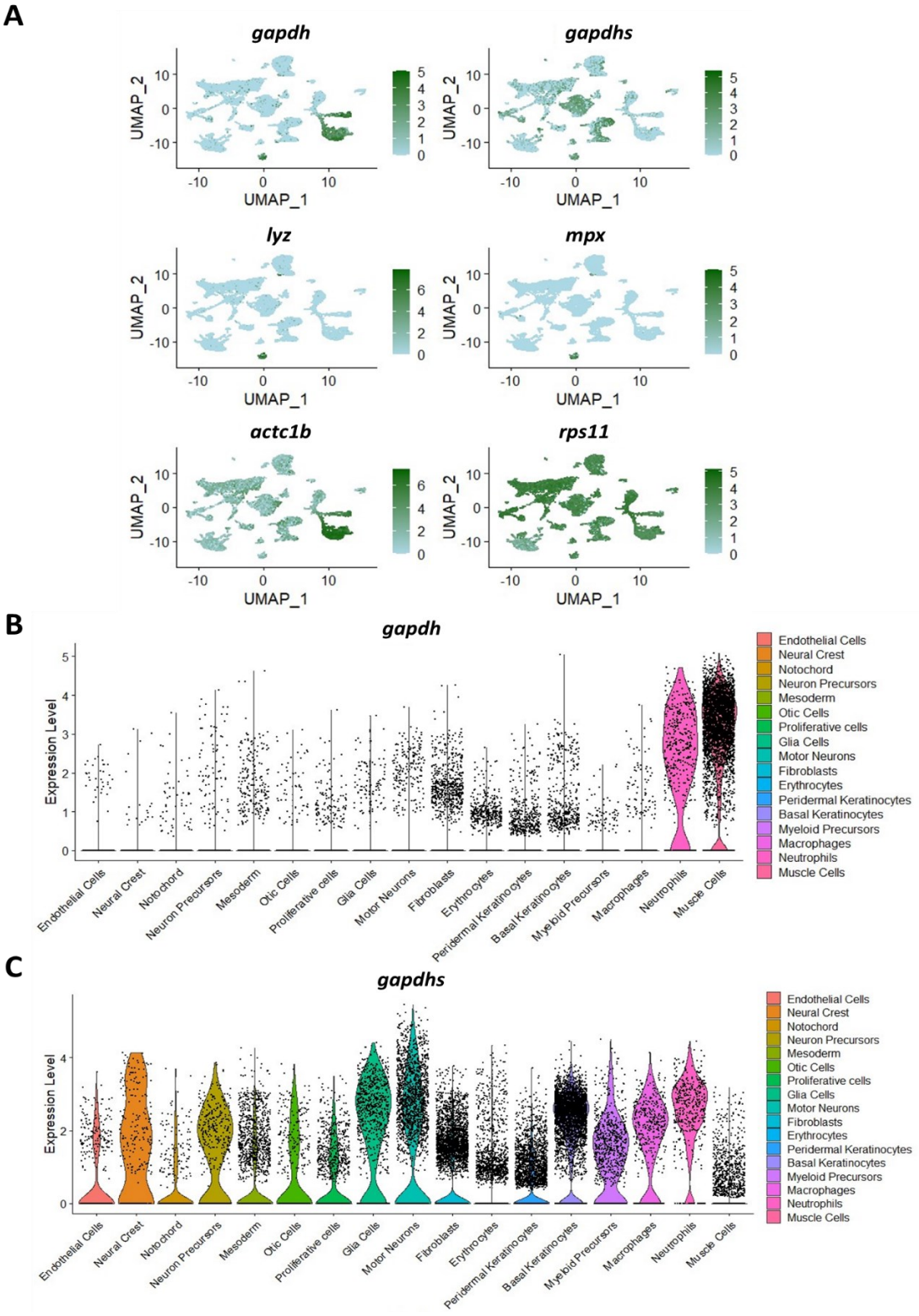

Figure 43. Expression profile of *gapdh* and *gapdhs* in the different tissues of 3 dpf larvae. (A) Single cell RNA-seq Feature Plot showed *gapdh* expression in muscle cell cluster (*actc1b*-overexpressing cells) and neutrophils cluster (*mpx* positive cells). The expression of *rps11* was used as a positive control of a housekeeping gene. **(B)** Violin Plot showing the expression of *gapdh* along the different clusters obtained. **(C)** Violin Plot showing the expression of *gapdhs* along the different clusters obtained. Each point represents the gene expression level in each cell analyzed.

larvae upon genetic inhibition of *gapdhs* independently of *gapdh*, while genetic inhibition of

gapdh alone resulted in slightly increased Gapdh protein amounts (Fig. 45D-E). In order to check the Gapdh enzymatic activity after the genetic inhibition of *gapdh* genes, a GAPDH enzymatic activity kit was used. The results showed that while genetic inhibition of *gapdh* alone and combined with *gapdhs* resulted in reduction Gapdh activity, the genetic inhibition of *gapdhs* did not affect the enzymatic activity of the protein (Fig. 45F-G).

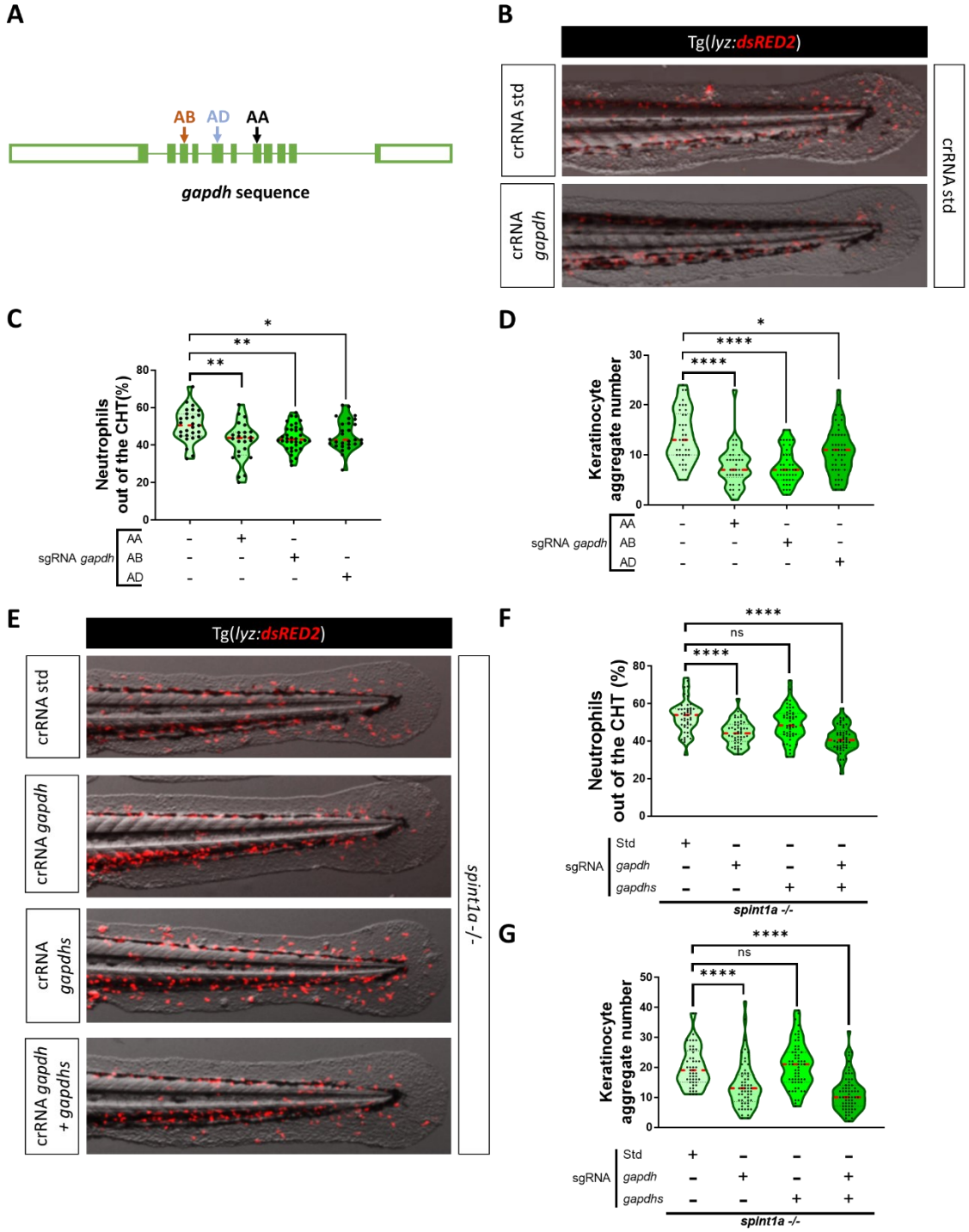

Figure 44. Genetic inhibition of *gapdh*, but not *gapdhs*, ameliorates chronic skin inflammation of Spint1a-deficient larvae. (A) Three different crRNAs, AA, AB and AD, were designed against *gapdh*

sequence in the seventh, third and fifth exon, respectively. **(B)** Representative merge images showing the skin and neutrophils of Spint1a-deficient larvae microinjected with a standard crRNA and the crRNA of *gapdh* AA in the presence of recombinant Cas9. **(C)** Neutrophil dispersion **(D)** Number of keratinocyte aggregates in the skin. **(E)** Representative merge images showing the skin and neutrophils of Spint1a-deficient larvae of 3 dpf microinjected with a standard crRNA, *gapdh* crRNA, *gapdhs* crRNA and the combination of both crRNAs of *gapdh* and *gapdhs*. **(F)** Number of keratinocyte aggregates in the skin. **(G)** Neutrophil dispersion. The mean ± SD for each group is shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. crRNA, CRISPR RNA; CHT, caudal hematopoietic tissue; std, standard; *gapdh*, glyceraldehyde-3-phosphate dehydrogenese; *gapdhs*, glyceraldehyde-3-phosphate dehydrogenese, spermatogenic; ANOVA, analysis of variance.

As Gapdh, but not Gapdhs, seemed to be involved in chronic skin inflammation in the Spint1a-deficient model. a protein sequence alignment was carried out between Gapdh (Q5XJ10 in Uniprot database) and Gapdhs (Q5MJ86 in Uniprot database). The alignment using Clustal Omega revealed an identity of 76.51% between both proteins, with a rather similar number of amino acids (Gapdh had 333 and Gapdhs 335 amino acids) (**Figure 46**).

In order to confirm the specificity of *gapdh* genetic inhibition, a rescue experiments using *gapdh* mRNA was performed. Moreover, to ascertain whether the effect of Gapdh on skin inflammation was mediated either by the canonical enzymatic function of the protein or by its translocation to the nucleus, a mutant version of *gapdh* mRNA, which encodes the mutant Gapdh-C150S, which was reported to impair Gapdh nitrosylation and nuclear translocation (T. Sen et al., 2018), was also used. While forced expression of the mRNA encoding wild type Gapdh was able to rescue the anti-inflammatory effects of genetic inhibition of *gapdh*/*gapdhs* in Spint1a-deficient larvae (**Figure 47A, B**), forced expression of the mRNA encoding Gapdh-C150S failed to do so (**Figure 47A, B**), despite both mRNAs restored Gapdh protein levels in Gapdh-deficient larvae (**Figure 47**C).

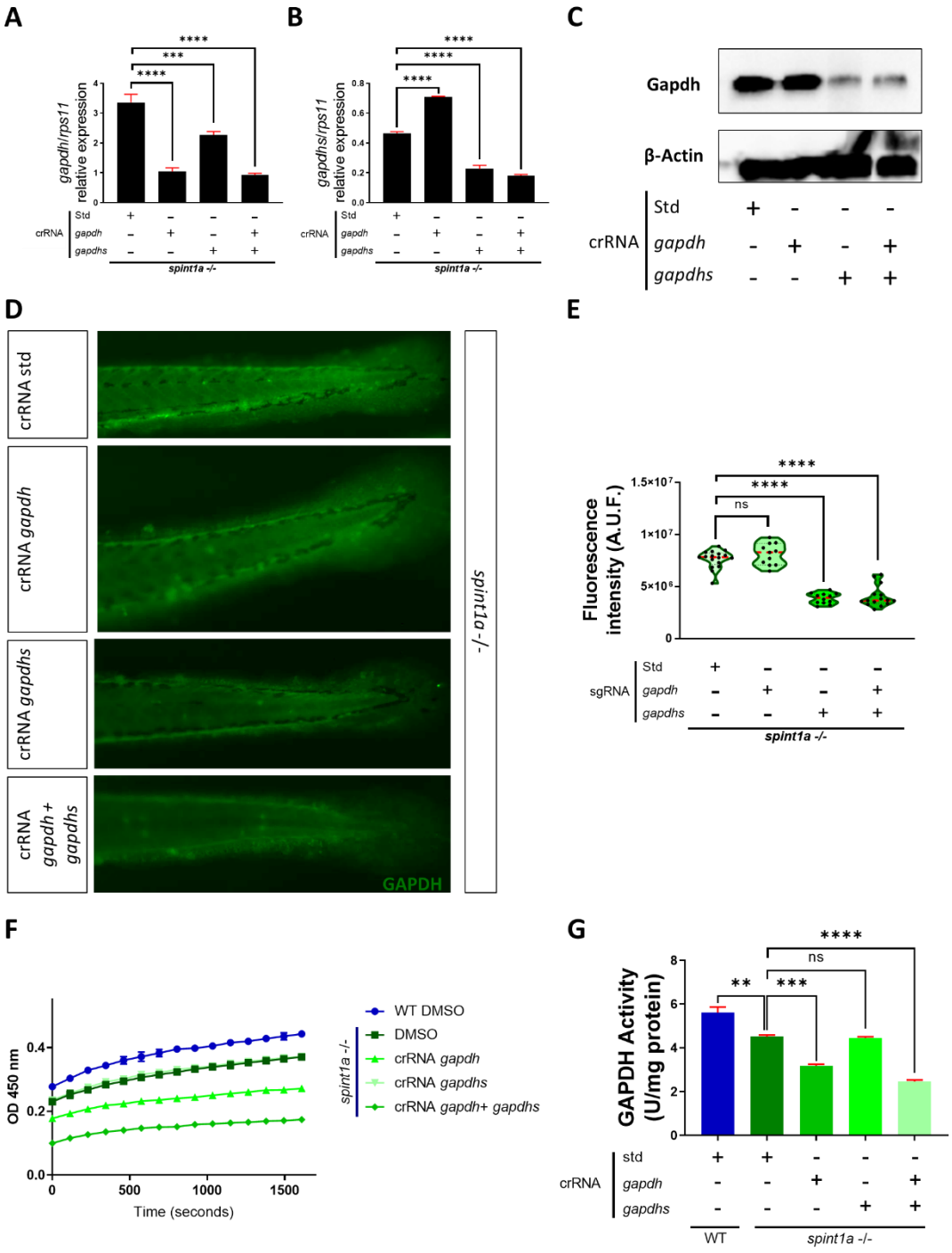

Figure 45. Genetic inhibition of *gapdhs*, but not of *gapdh*, results in reduced protein amount and enzymatic activity of Gapdh. (A, B) Transcript levels of *gapdh* (A) and *gapdhs* (B) after genetic inhibition of *gapdh* and/or *gapdhs*, assayed by RT-qPCR in 3 dpf Spint1a-deficient larvae. (C) Representative Western blot of Gapdh protein amounts in 3 dpf Spint1a-deficient larvae after genetic inhibition of *gapdh* and/or *gapdhs*. **(D, E)** Representative images showing Gapdh immunohistochemistry in Spint1a-deficient larvae upon genetic inhibition of *gapdh* and/or *gapdhs* (D) and quantitation Gapdh protein amount (E). **(F, G)** Gapdh enzymatic activity in 3 dpf wild type and Spint1a-deficient larvae. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. **p≤0.01; ***p≤0.001; ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; std, standard; crRNA, CRISPR RNA; WT, wildtype; *gapdh*, glyceraldehyde-3-phosphate dehydrogenese; *gapdhs*, glyceraldehyde-3-phosphate dehydrogenese, spermatogenic; OD, Optical Density; U, Enzymatic activity units.

```
Percent Identity  Matrix - created by Clustal2.1

  1: sp|Q5XJ10|G3P_DANRE   100.00   76.51
  2: sp|Q5MJ86|G3P2_DANRE   76.51  100.00

sp|Q5XJ10|G3P_DANRE
sp|Q5MJ86|G3P2_DANRE

sp|Q5XJ10|G3P_DANRE       --MVKVGINGFGRIGRLVTRAAFLTKKVEIVAINDPFIDLDYMVYMFQYDSTHGKYKGEV  58
sp|Q5MJ86|G3P2_DANRE      MSELCVGINGFGRIGRLVLRAC-LQKGIKVTAINDPFIDLQYMVYMFKYDSTHGRYKGEV  59
                             : ************ **. * * :::.*********:******:******:*****

sp|Q5XJ10|G3P_DANRE       KAEGGKLVIDGHAITVYSERDPANIKWGDAGATYVVESTGVFTTIEKASAHIKGGAKRVI  118
sp|Q5MJ86|G3P2_DANRE      HMEDGKLIVDGQAISVFQCMKPAEIPWGDAGALYVVESTGVFLSIEKASAHIQGGAKRVV  119
                          : *.***::**:**:*:.  .**:* ****** ********* :********:******:

sp|Q5XJ10|G3P_DANRE       ISAPSADAPMFVMGVNHEKYDN-SLTVVSNASCTTNCLAPLAKVINDNFVIVEGLMSTVH  177
sp|Q5MJ86|G3P2_DANRE      VSAPSPDAPMFVMGVNQDKYDPSSMTIVSNASCTTNCLAPLAKVIHDNFGIEEALMTTVH  179
                          :**** **********::***  *:*:******************:*** * *.**:***

sp|Q5XJ10|G3P_DANRE       AITATQKTVDGPSGKLWRDGRGASQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPN  237
sp|Q5MJ86|G3P2_DANRE      AYTATQKTVDGPSAKAWRDGRGAHQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPVAD  239
                          * **********.* ******* ********************************. :

sp|Q5XJ10|G3P_DANRE       VSVVDLTVRLEKPAKYDEIKKVVKAAADGPMKGILGYTEHQVVSTDFNGDCRSSIFDAGA  297
sp|Q5MJ86|G3P2_DANRE      VSVVDLTCRLTRPASYANIKESVKKAAHGPMKGILGYTEDSVVSSDFVGDTHSSIFDAGA  299
                          ******* ** :**.* :**: ** **.**********..***:** ** :********

sp|Q5XJ10|G3P_DANRE       GIALNDHFVKLVTWYDNEFGYSNRVCDLMAHMASKE  333
sp|Q5MJ86|G3P2_DANRE      GISLNDNFVKLISWYDNEFGYSHRVADLLMYMHSKE  335
                          **:***:****:*********:**.**: :* ***
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sp|Q5XJ10|G3P_DANRE 0.23494
sp|Q5MJ86|G3P2_DANRE 0.23494

Figure 46. Protein sequence alignment of zebrafish Gapdh and Gapdhs. Aligment using Clustal Omega showed a strong identity of 76,51% between both proteins, observed along all the sequence of the proteins, which showed also a similar number of amino-acids in their polypeptidic chain, being 333 in the case of Gapdh and 335 in the case of Gapdhs. An asterisk (*) indicates positions which have a conserved residue; A colon (:) indicates conservation between groups of strongly similar properties, equivalent to scoring > 0.5 in the Gonnet PAM 250 matrix; A period (.) indicates conservation between groups of weakly similar properties as below - roughly equivalent to scoring =< 0.5 and > 0 in the Gonnet PAM 250 matrix (https://www.ebi.ac.uk/seqdb/confluence/display/THD/Help+-+Clustal+Omega+FAQ).

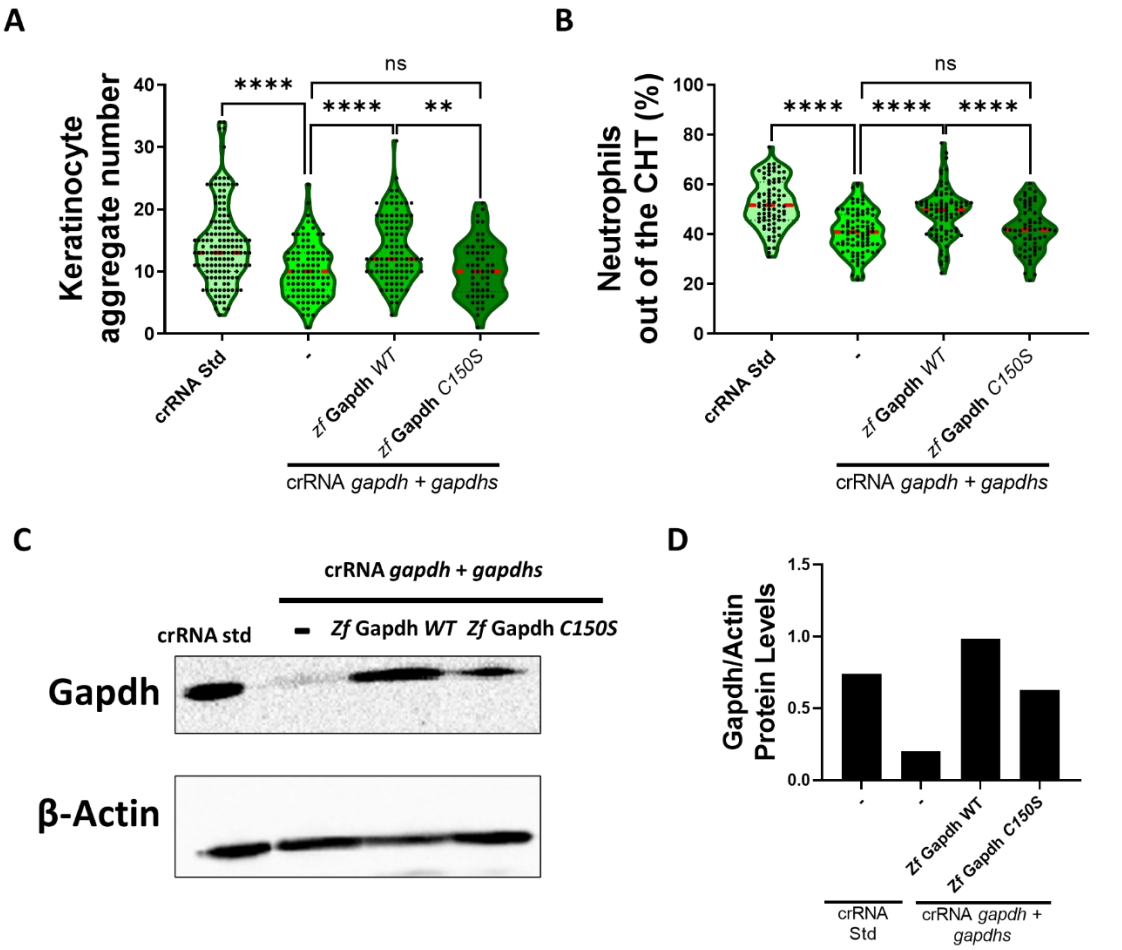

Figure 47. Wild type Gapdh, but not nuclear translocation defective Gapdh-C150S, rescues the anti-inflammatory effects of Gapdh/Gapdhs deficiency. (A-C) Gapdh/Gapdhs-deficient larvae were forced to express mRNAs encoding either wild type Gapdh or Gapdh-C150S and the number of keratinocyte aggregates (A), neutrophil dispersion (B) and Gapdh protein amounts were analyzed at 3 dpf. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA and Tukey multiple range test. **p≤0.01; ***p≤0.001; ****p ≤ 0.0001. Std, standard; crRNA, CRISPR RNA; WT, wild type; gapdh, glyceraldehyde-3-phosphate dehydrogenese; gapdhs, glyceraldehyde-3-phosphate dehydrogenese, spermatogenic; CHT, caudal hematopoietic tissue.

As the scRNA-seq data showed that *gapdh* was only expressed in muscle cells and neutrophils, an important role of neutrophils in the development and progression of chronic skin inflammation has been broadly studied, and GAPDH has been recently reported to be involved in the regulation or activation of some neutrophil functions, such as NETosis, cell death or immunomodulation (Y. Li et al., 2023; Taniguchi-Ponciano et al., 2021), we next focused our attention in these cells. The neutrophil cluster containing wild type and Spint1a-deficient neutrophils was selected and recluster in the scRNA-seq, obtaining a total of eight different neutrophil subclusters (**Figure 48**). A higher number of neutrophils were obtained from the Spint1a-deficient larvae than from wild type larvae. In addition, wild type larvae were mainly distributed in the cluster 2, although some of them were also observed in other clusters with the

exception of cluster 0 and 6, that were mainly formed by Spint1a-deficient neutrophils (**Figure 48**).

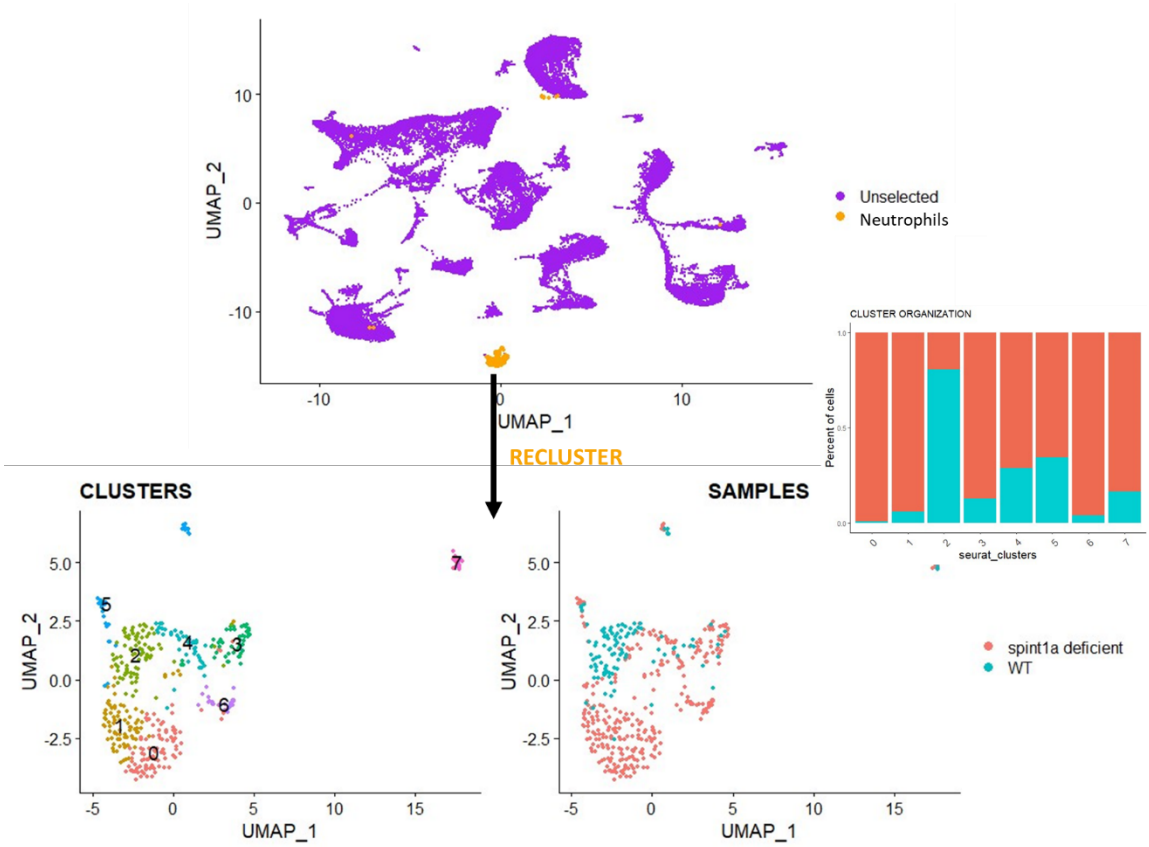

Figure 48. Neutrophil reclustering showed eight different neutrophil subclusters. Neutrophil population (highlighted in orange) was selected based on the expression of the markers *mpx* and *lyz* (Fig. 15), and they were reclustered. The reclustering generated eight different clusters, numbered from 0 to 7. Histogram shows cluster organization, indicating the percentage of cells from each sample that constitute each cluster. UMAP shows neutrophils grouped by clusters (left) and sample origin (right). WT, wildtype; UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

Neutrophils subclusters were analyzed and classified according to their different gene expression profiles. Gene enrichment analysis using Gene Ontology: Biological Process information showed no significant pathways associated to the cluster 1 and cluster 2. Cluster 0 showed enriched expression of genes involved in cytoskeleton organization associated to cell migration (*mmp13a* and *mmp9*), cluster 3 specific genes involved in neutrophil development (*pmp22a* or *col5a2a*), cluster 4 genes associated with RNA processing, although the results obtained were not statistically significant and its main markers, *myb* and *csf1rb*, were not involved in this pathway. Neutrophil cluster 5 was enriched in genes involved in cell adhesion, such as *epcam*, while cluster 6 was integrated by proliferating neutrophils, whose main significant pathways were related to cell cycle and mitosis, such as *cdk1* and *mki67*. Finally, cluster 7 was integrated by neutrophils expressing erythroid-like genes, such as *hemgn* or *cahz*, suggesting that they were erythromyeloid progenitors (**Figure 49**).

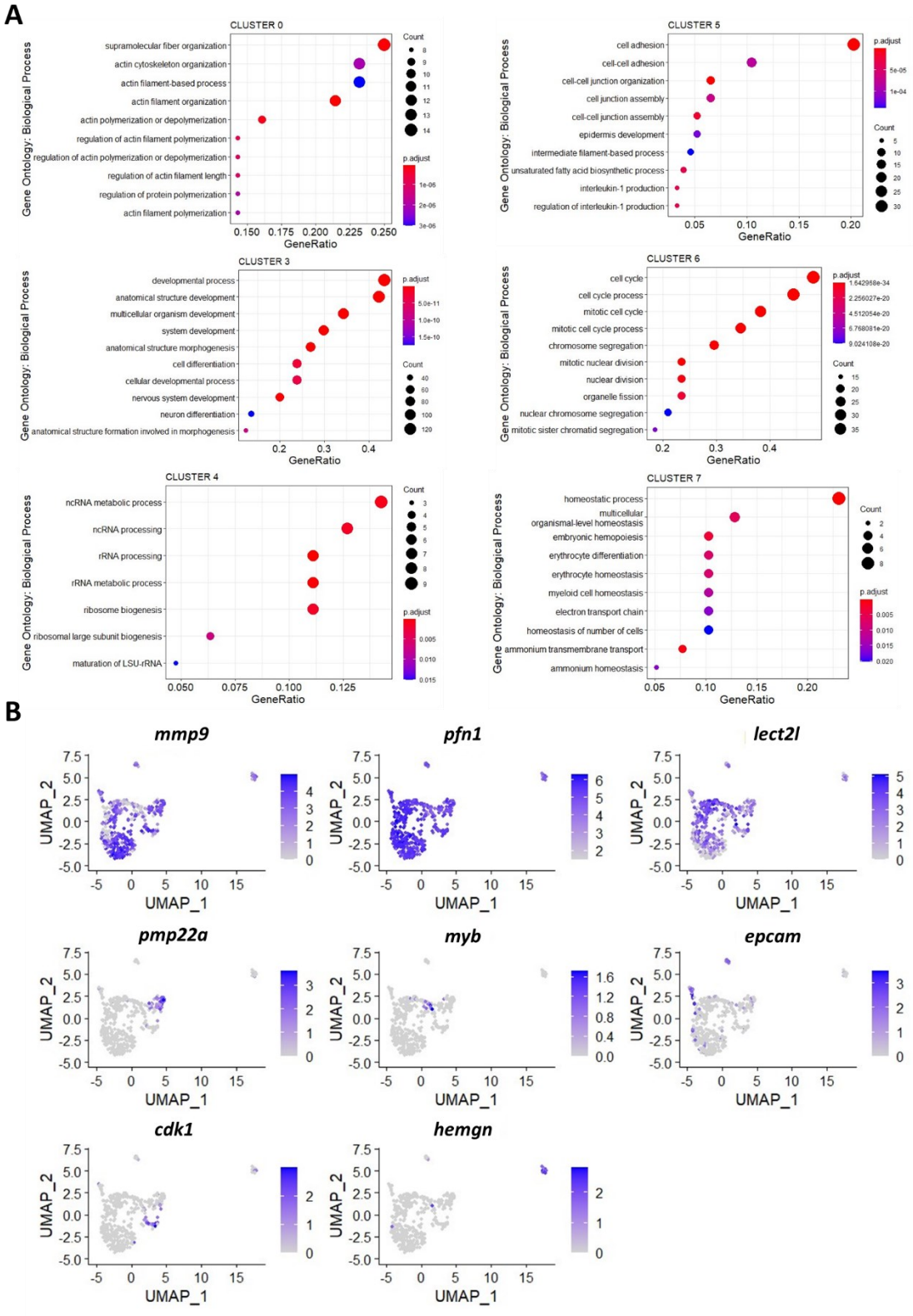

Figure 49. Characterization of neutrophil subclusters obtained by scRNA-seq analysis. (A) Dotplots showing the 10 most significantly altered Gene Ontology: Biological Process pathways in the neutrophil subclusters 0, 3, 4, 5, 6 and 7 are shown. No significant pathways were obtained for clusters 1 and 2. **(B)** FeaturePlot showing the most differentially expressed gene of each neutrophil subcluster. *mmp9, matrix metalloproteinase 9; pfn1, perforin-1; lect2l, leukocyte cell derived chemotaxin 2-like; pmp22a, peripheral myelin protein 22a; myb, v-myb avian myeloblastosis viral oncogene homolog; epcam, epithelial cell adhesion molecule; cdk1, cyclin dependent kinase 1; hemgn, hemogen.*

· Discussion

In this chapter, we have tried to better understand the different processes that are taking place in the Spint1a-deficient larvae that can be related to the development of its characteristic chronic skin inflammation, and specifically the relevance of nuclear translocation of Gapdh in skin inflammation. scRNA-seq analysis using pooled tails of 3 dpf wild type and Spint1a-deficient larvae revealed 17 different cell populations (**Figure 40**). The powerful scRNA-seq technology allowed us to analyze the different cluster individually and established the gene expression profile of each population in wild type and Spint1a-deficient larvae, as it was done with peridermal, basal keratinocytes, and neutrophils. Moreover, the data can be used to reveal novel specific cell markers or predicted novel functions during inflammation, as it was done for neutrophils.

In the previous chapter we observed some specific features of the inflamed skin of Spint1a-deficient larvae, such as the appearance of keratinocyte aggregates associated to their hyperproliferation and increased death, and the higher levels of Gapdh, despite this enzyme has been widely considered a housekeeping protein, and neutrophil recruitment to the skin. For these reasons, we firstly focused on the study of keratinocytes. Two different clusters of keratinocytes were observed, namely peridermal keratinocytes, characterized by the expression of genes like *icn2* or *krt17*, and basal keratinocytes, characterized by the expression of *tp63*, *cldni* and *krt91*. These two populations have been widely studied in other zebrafish models of skin diseases, like for example the *psoriasis* model, where the contribution of each layer to inflammation and malignant transformation has been established (Hatzold et al., 2016; Webb, Driever, & Kimelman, 2008).

Zebrafish keratinocytes from the periderm showed differential expression of genes involved in the biosynthesis and metabolism of fatty acids, eicosanoids or prostaglandins. This not surprising, since eicosanoids, and prostaglandins in particular, have been associated to keratinocyte proliferation, inflammation or cell death (Ashcroft, Mahammad, Midtun Flatekval, Jullumstro Feuerherm, & Johansen, 2020; Honda et al., 2009; Shou et al., 2021), and they have been linked to chronic skin inflammatory disorders, such as psoriasis or atopic dermatitis (Kanda, Mitsui, & Watanabe, 2004; Sawada et al., 2019; Shou et al., 2021). Furthermore, fatty acid metabolism has also been associated to chronic skin inflammation, and changes in fatty acid metabolism and lipid dysfunction have been involved in the exacerbation of psoriasis (Sorokin et al., 2023; Zhao et al., 2023). These data offer new insights into the skin inflammation process and pave the way to study future studies aimed at identifying novel prognosis and therapeutic biomarker. To achieve this, complementary metabolomics and lipidomics studies in the Spint1a-

deficient line could offer more information about how these pathways are altered in chronic skin inflammation. With this data, new treatments based on fatty acids or eicosanoids, or already FDA/EMA-approved inhibitors of the different pathways, could be rapidly tested in the Spint1a-deficient larval model to evaluate the effect in the skin inflammation and to get enough information to move towards clinical trials in human patients.

Zebrafish basal keratinocytes are involved in the generation of the differentiated keratinocytes which form all strata in larvae and adult zebrafish (R. T. Lee, Asharani, & Carney, 2014; Yin, Hu, & Li, 2023), and they have been involved in development, like in actinotrichia and fin formation (Kuroda, Iwane, & Kondo, 2018), tumor-promoting processes (Hatzold et al., 2016), or entosis and apical cell extrusion in chronic skin inflammation (Armistead et al., 2020). Our scRNA-seq analysis revealed that the main pathways altered in basal keratinocytes of Spint1a-deficient larvae were related to developmental processes, being not surprising according to the known roles played by zebrafish basal keratinocytes discussed above. Importantly, these results also suggest that basal keratinocytes are involved in the formation of the keratinocytes aggregates characteristic of the model due to the alteration of normal development. To do so, analysis and subcluster of these keratinocytes would be required to identify and characterize the keratinocytes present in the aggregates, and responsible of their formation. Although this is out of ethe scope of this study, R packages, like CellChat or CommPath, could be applied to the different keratinocyte clusters in order to study the cell-cell communication and found altered signaling patterns in the Spint1a-deficient larvae that could be responsible of this epithelial disruption (Jin et al., 2021; H. Lu et al., 2022).

After the analysis of the different keratinocyte clusters and their gene expression profiles in wild type and Spint1a-deficient larvae, no obvious associations to Gapdh function was obtained. Therefore, we analyzed the expression of *gapdh* through the different clusters in 3 dpf larvae and found that 2 *gapdh* genes, named *gapdh* and *gapdhs*, were present in zebrafish, as occurs in other vertebrates including humans. On the one hand, in 3 dpf zebrafish larvae, *gapdh*, which was supposed to be the main one, was only expressed in neutrophils and muscle cells. The expression on muscle cells might be related to the high rate of metabolism of these cells to allow the larvae mobility but this needs further studies. However, the specific expression of *gapdh* in neutrophils was unexpected. On the other hand, *gapdhs* was rather ubiquitously expressed, with the exception of muscle cells, where its transcript levels were much lower than in the rest of the tissues.

In human, GAPDH has a molecular mass of 36 kDa and contains 335 amino acids, while GAPDHS has a molecular mass of 44,5 kDa and contains 408 amino acids, with a specific 72-amino acid length N-terminus (Elkina et al., 2010; Gill et al., 2022). Unfortunately, this difference observed in human is not present in zebrafish, where Gapdh had a 333 amino acid sequence, while Gapdhs had 335 amino acids. Furthermore, the identity of both proteins is 76,51% and the showed key residues involved in non-canonical functions, including the C152 (C150 in zebrafish), involved in nuclear translocation; G10, involved in nuclear GAPDH-PARP1 interaction; and Y41, involved in GAPDH phosphorylation, among others. The only residue not conserved in Gapdhs compared to Gapdh is K259, which in Gapdhs is substituted by glutamic acid. As regards GAPDH nuclear export after carrying out its nuclear function, although no specific residues have been reported to be involved in this process, some studies have reported that nuclear export is mediated by Exportin-1 and acts as a protective mechanism against GAPDH-mediated cell death (Schmitz, 2001; Schmitz, Dutine, & Bereiter-Hahn, 2003). Recently, AMPK was related to GAPDH nuclear export (Sohn, Kwak, Rhim, & Yeo, 2022), but all these studies are controversial, because demonstrating whether these pathways trigger nuclear export or impaired nuclear import is challenging.

CRISPR-Cas9 technology was then used to inhibit both *gapdh* genes. These studies revealed that *gapdh*, but not *gapdhs*, was involved in the development of the chronic skin inflammation characteristic of the Spint1a-deficient model. More importantly, rescued experiments with mRNAs encoding wildtype Galpdh and nuclear translocation-deficient Gapdh-C150S unequivocally demonstrated that Gapdh nuclear translocation, rather than its canonical glycolytic activity, promotes chronic skin inflammation. Notably, mRNA expression levels analyzed by RT-qPCR showed decreased levels of *gapdh* and *gapdhs* mRNA after genetic inhibition of the respective genes. The most surprising result obtained was the compensatory effect of the genetic inhibition of *gapdh* by *gapdhs*. This result correlated with the Western blot assay, in which inhibition of *gapdhs* resulted in reduced protein levels of Gapdh, while the inhibition of *gapdh* showed no apparent changes in the protein levels. This result can be explained by the inability of the anti-Gapdh antibody used to discriminate between Gapdh and Gapdhs because of their strong sequence similarity and the higher levels of Gapdhs, which might mask the changes in Gapdh. In addition, the compensatory effect of *gapdhs* after the genetic inhibition of *gapdh* could also explain this result. Furthermore, it was unexpectedly found that Gadph activity declined in Gapdh-deficient, but no in Gapdhs-deficient larvae, despite *gapdhs* mRNA levels were higher than those of *gapdh* and ubiquitously expressed, and genetic inhibition of *gapdhs* resulted in reduced Gapdh protein amounts. A possible explanation might be the specific expression of *gapdh* in the muscle. Myoblast and muscle cells are one of the cells with

the highest glycolytic rate (Bittner et al., 2010), and GAPDH is one of the most important enzymes in glycolysis (Kornberg et al., 2018). It is tempting to speculate, therefore, that zebrafish Gapdh has much higher glycolytic activity than Gapdhs in order to satisfy the high energetic demand of the skeletal muscle. Determination of Gapdh activity in sorted cells of *Tg(actc1b:GFP)* (Higashijima, Okamoto, Ueno, Hotta, & Eguchi, 1997) and/or of purified recombinant proteins could help to clarify this issue.

We next turned our attention to neutrophils, since Gapdh was specifically expressed in neutrophils and muscle cells. We, therefore, hypothesized that Gapdh nuclear translocation alters neutrophil function that, in turn, contributes to skin inflammation in Spint1a-deficient larvae. Reclustering of neutrophil population obtained with scRNA-seq analysis revealed eight different neutrophil subclusters of which wild type neutrophils were mainly grouped in cluster 2, while neutrophils from Spint1a-deficient larvae were more represented in the rest of the clusters, being almost specific in clusters 0 and 6. An interesting observation was that cluster 0 and cluster 1 presented markers related to actin and cytoskeleton organization, which suggest that they represent migrating neutrophils to the inflamed area. Furthermore, cluster 3, which was enriched in many genes involved in development, and cluster 4, which enriched expression of the HSPC marker *myb*, which is also involved in neutrophil maturation (Z. Huang et al., 2021), together with genes involved in rRNA processing, suggest that they represent the neutrophils expanded in the CHT in response to skin inflammation. Cluster 6 is also related to neutrophil expansion in the Spint1a-deficient model, since it represents neutrophils which expressed a broad number of genes involved in cell cycle progression and proliferation (35 genes), such as *cdk1*, *mki67* or *kif11.* As they did not express HSPC markers, it is tempting to speculate that this cluster represent neutrophils generated by emergency granulopoiesis, a process triggered by an inflammatory process in which immature neutrophils migrate to the inflamed tissues and maintain some immature characteristics, such as proliferation (Gullotta et al., 2023; A. J. Kwok et al., 2023; I. Kwok et al., 2020; Manz & Boettcher, 2014). Immature neutrophils have been recently reported as important factors in the development of some diseases (Gullotta et al., 2023; A. J. Kwok et al., 2023; I. Kwok et al., 2020; Manz & Boettcher, 2014), what makes this cluster, together with clusters 3 and 6, promising for its future study in this model.

Unexpectedly, we found that cluster 5 was enriched in genes encoding cell adhesion and cell-cell junction molecules, such as *cav2*, suggesting that they may represent either neutrophils in the process of extravasation or already interacting with keratinocyte aggregates. Finally, the identify of neutrophils of cluster 7, mainly integrated by neutrophils from Spint1a-deficient larvae, is also difficult to infer, since they were enriched in genes usually expressed in erythrocyte, such as

hemgn or *cahz*. They might represent erythromyeloid precursors that could have also been expanded in response to skin inflammation.

Chapter 3: Neutrophil Gapdh exacerbates chronic skin inflammation of Spint1a-deficient larvae

· Results

In order to evaluate the role of neutrophils in chronic skin inflammation, the firstly ablated neutrophils using the Gal4/UAS system (Halpern et al., 2008). To do so, the zebrafish line *TgBAC(mpx:GAL4-VP16)* was crossed with the line *Tg(UAS-E1B:NTR-mCherry)* (Davison et al., 2007; Robertson et al., 2014). The offspring would express NTR-mCherry in neutrophils thanks to the neutrophil-specific *mpx* promoter and *spint1a* was inhibit by microinjecting the fertilized eggs with *spint1a* crRNA/Cas9 complexes. Some of the larvae were treated with metronidazole, a compound that is going to be processed by the nitrorreductase (NTR) expressed in the neutrophils, generating a toxic compound that kill them (**Figure 50A**). At 3 dpf, the number of keratinocyte aggregates of untreated and metronidazole-treated larvae was evaluated. Metronidazol treatment drastically ablated neutrophils (**Figure 50B**) and this led to reduced number of keratinocyte aggregates (**Figure 50C**). Metronidazol failed to reduce keratinocyte aggregates in Spint1a-deficient larvae that did not express NTR (data not shown).

To further confirm the pro-inflammatory effect of neutrophils in chronic skin inflammation, neutrophilia was generated by forcing the expression of a mRNA encoding zebrafish Csf3a (Mehta & Corey, 2021). The results showed that the number of keratinocyte aggregates in the skin of *csf3a*-microinjected Spint1a-deficient larvae resulted not only in increased number of neutrophils but also in keratinocyte aggregates (**Figure 51A-C**).

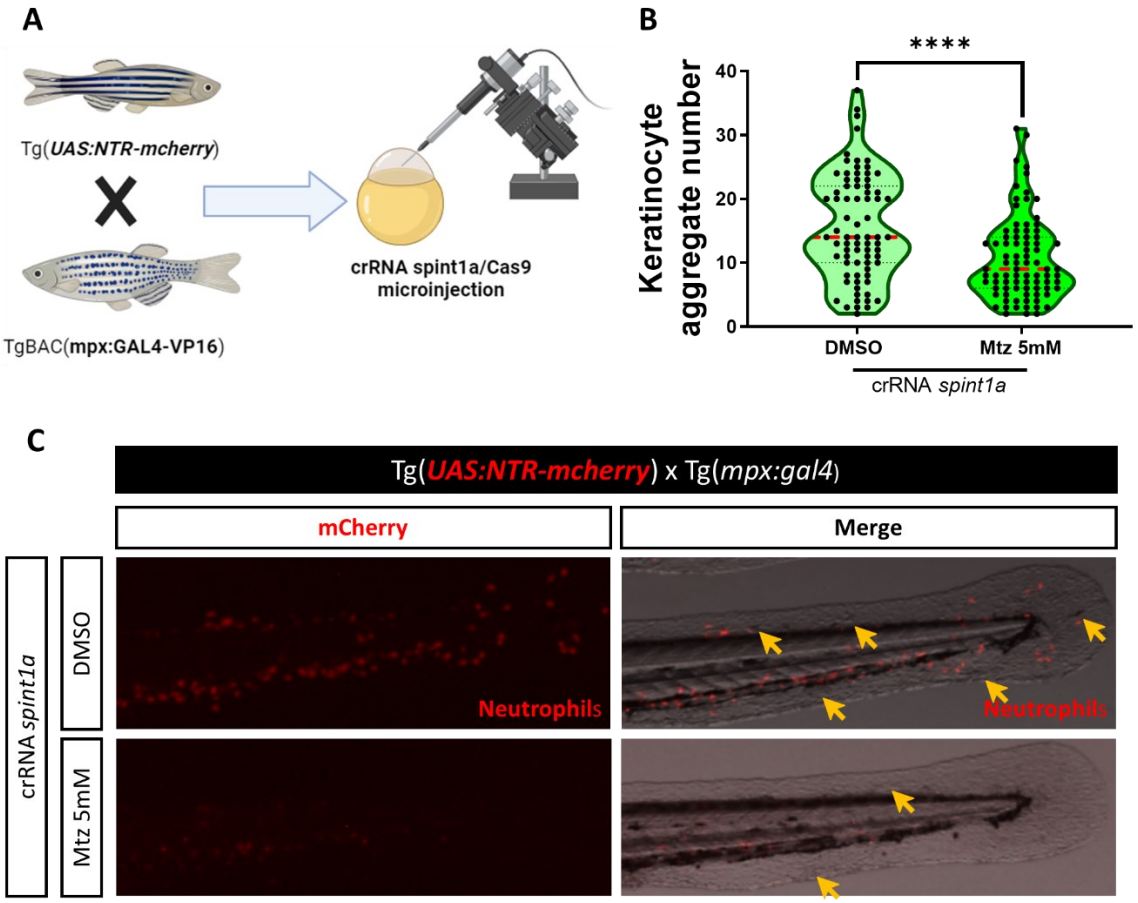

Figure 50. Neutrophil ablation alleviates chronic skin inflammation. (A) The lines *TgBAC(mpx:GAL4-VP16*) and *Tg(UAS-E1B:NTR-mCherry)* were crossed and the embryos were microinjected with *spint1a* crRNA in the presence of recombinant Cas9 at one-cell state of development. At 1dpf embryos were dechorionated and treated with DMSO and 5 mM metronidazole (MTZ) for 48 h. Three dpf larvae were imaged and the phenotype was quantified. **(B, C)** Keratinocyte aggregate number (B) and representative merged images showing skin and neutrophils are shown. Yellow arrows label keratinocyte aggregates in the skin. Each dot represents one individual, and the mean ± SEM for each group is also shown. p-Values were calculated using t test. ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; std, standard; crRNA, CRIPSR RNA; Mtz, metronidazole.

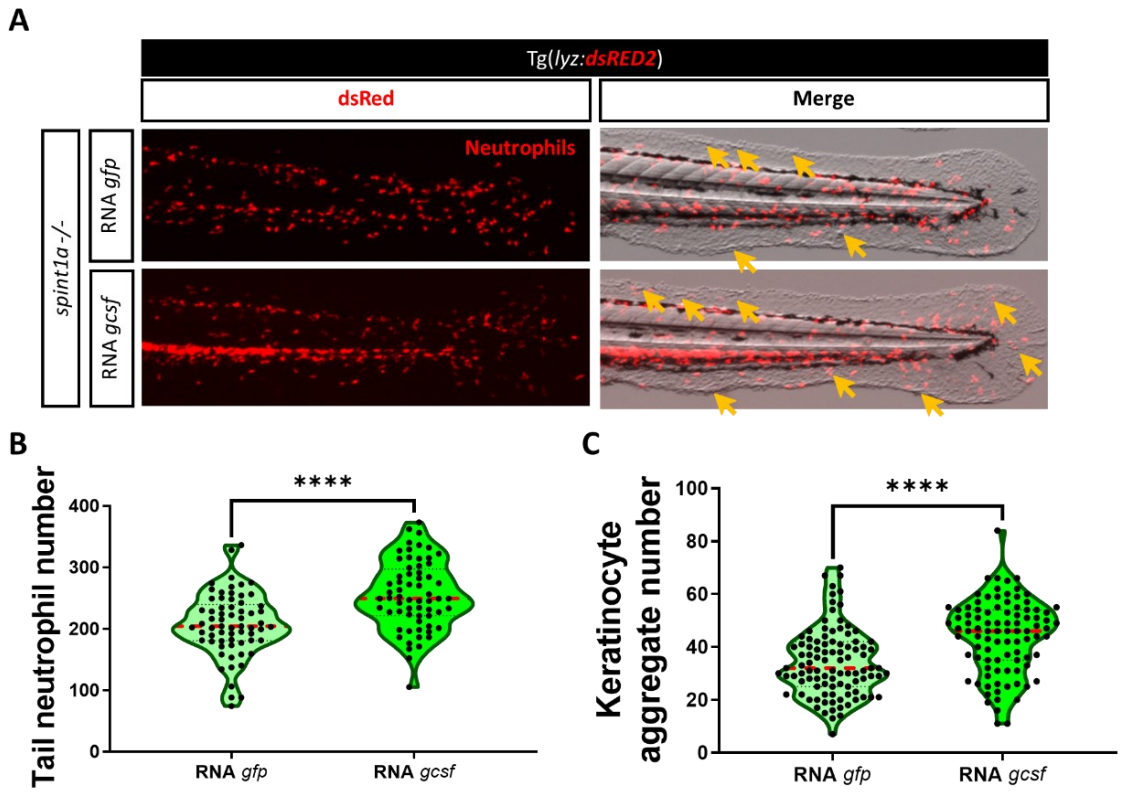

Figure 51. Csf3a.driven neutrophilia exacerbates chronic skin inflammation. (A) Representative merged images showing skin and neutrophils in Spint1a deficient larvae microinjected with *gfp* (control) and *csf3a* mRNAs. **(B, C)** Number of neutrophils (B) and keratinocyte aggregates (C). Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ****p ≤ 0.0001. *gfp*, green fluorescent protein; *csf3a*, Colony stimulating factor 3a (Granulocyte colony stimulating factor).

We next tried to stablish a correlation between the number of keratinocyte aggregates and the number of neutrophils in the different larvae analyzed. *Gfp* mRNA-microinjected control Spint1a-deficient larvae were analyzed, and it was observed a strong positive correlation between the number of neutrophils and the number of keratinocyte aggregates (**Figure 52A**). The same result was observed in the larvae microinjected with *csf3a* mRNA (**Figure 52B**). Moreover, skin-infiltrated neutrophils were analyzed and also correlated to the number of keratinocyte aggregates (**Figure 52C-D**).

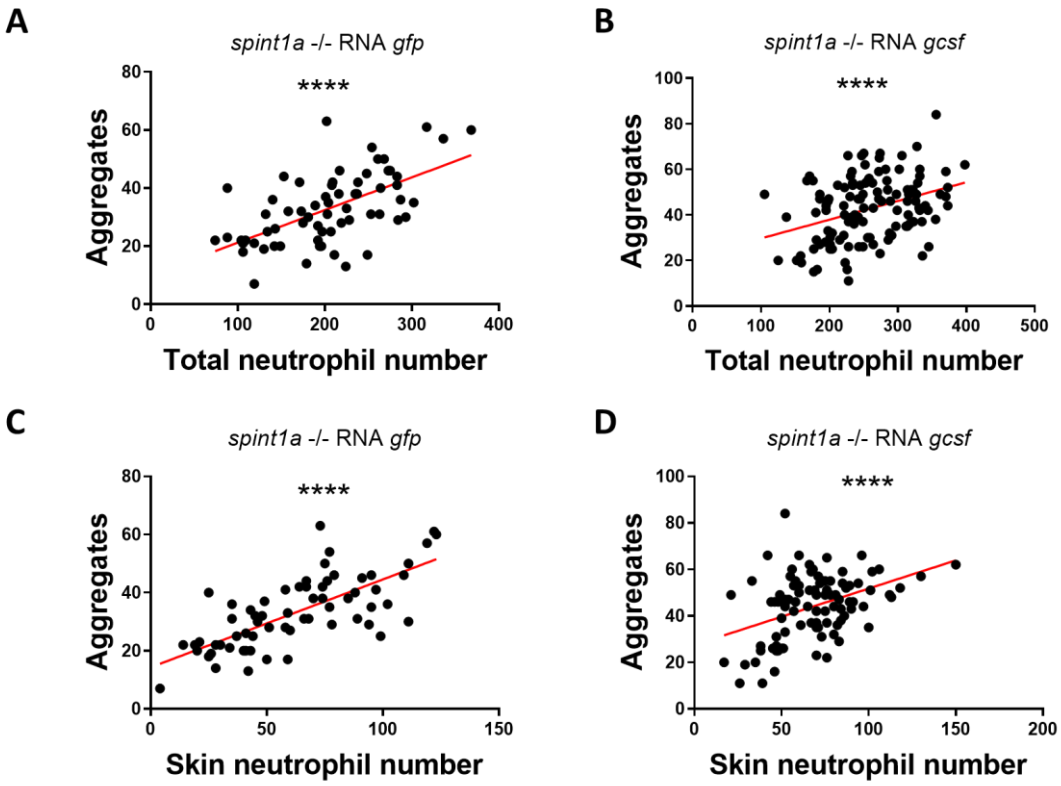

Figure 52. The number of neutrophil and keratinocyte aggregates shows strong positive correlation. (A, B) Correlation between the number of keratinocyte aggregates and the number of neutrophils in Spint1a-deficient larvae microinjected with the mRNA of *gfp* (A) or *csf3a* (B). **(C, D)** Correlation between the number of neutrophils infiltrated in the skin and the number of keratinocyte aggregates Spint1a-deficient larvae microinjected with *gfp* or *csf3a* mRNAs. Each dot represents one individual whose number of neutrophils (total or in the skin) and number of aggregates was quantified. p-Values were calculated using correlation. ****p ≤ 0.0001. *gfp*, green fluorescent protein; *csf3a*, colony stimulating factor 3a.

After confirming the negative role of neutrophils in chronic skin inflammation, our new objective was to go deeper into the neutrophil behavior in the skin of the Spint1a-deficient larvae by using laser confocal microscopy. Firstly, using a transgenic line with the keratinocytes tagged with GFP, *Tg(krt4:GFP)* (Z. Gong et al., 2002), two different neutrophil behaviour were observed: some of them were randomly circulating in the skin, while others were directly interacting with the keratinocyte of the aggregates (**Figure 53A-B**).

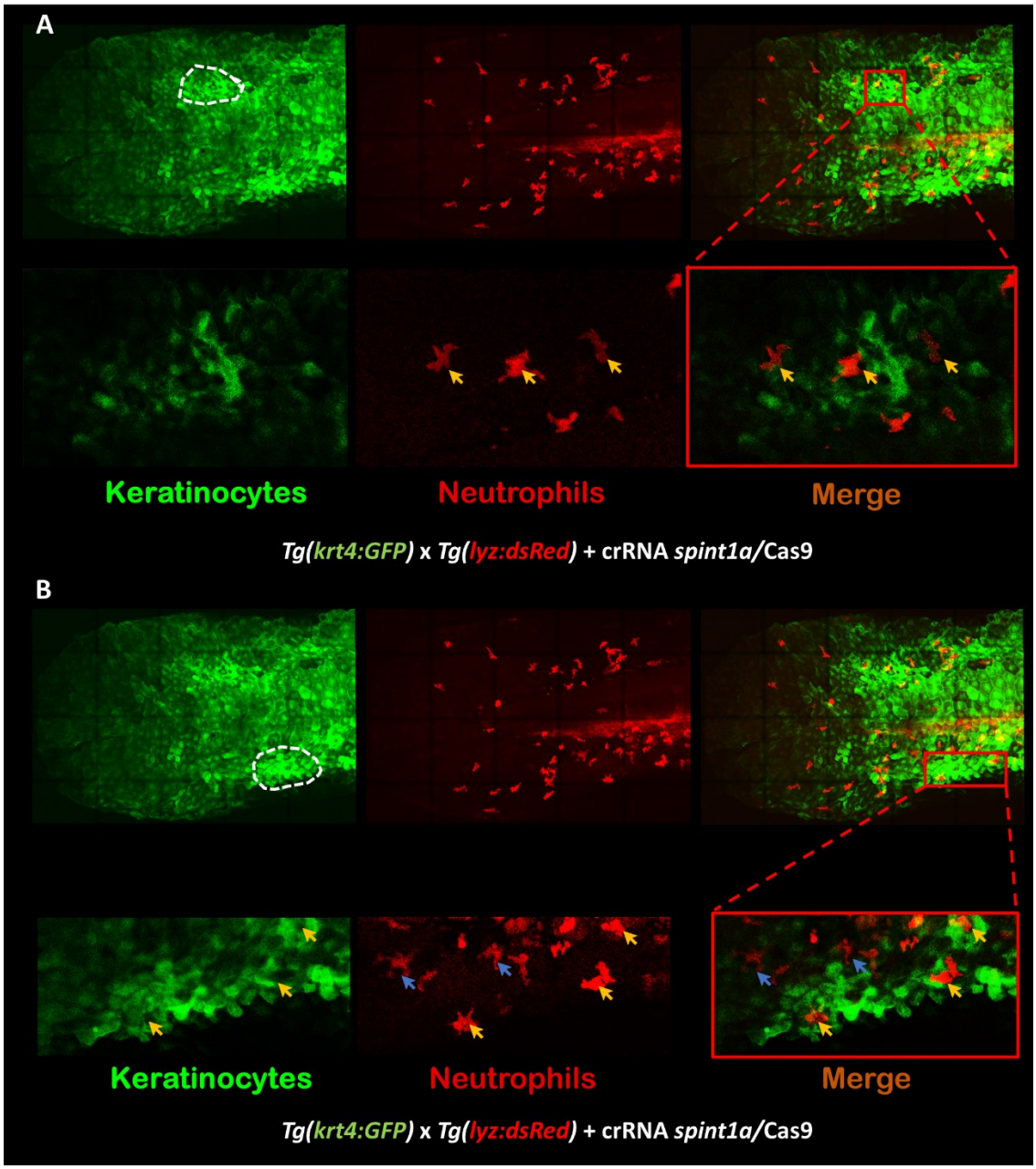

Figure 53. Neutrophil and keratinocyte interaction in the inflamed skin of the Spint1a-deficient larvae. Representative images obtained with a Stellaris Confocal Microscope from Tg(*krt4:GFP; lyz:dsRED*) larvae of 3 dpf microinjected with *spint1a* crRNA/Cas9 complexes at the one-cell stage. **(A)** Neutrophils interacting with the keratinocytes of a selected aggregate (dotted line) are indicated with yellow arrows. **(B)** Neutrophils interacting with the keratinocyte of a selected aggregate (dotted line) are indicated with yellow arrows, while the non-interacting neutrophils are indicated with blue arrows. *Krt4, keratin 4*; crRNA, CRISPR RNA; *lyz, lysozyme*.

In order to study the neutrophil behavior in this model, time-lapse imaging using a Spinning-disk confocal microscope were used. One of the most striking results obtained was the interaction between neutrophils and the keratinocyte of the aggregated and of neutrophils suffering cell division in the proximity of the keratinocyte aggregates (**Figure 54**; Supplementary video 1).

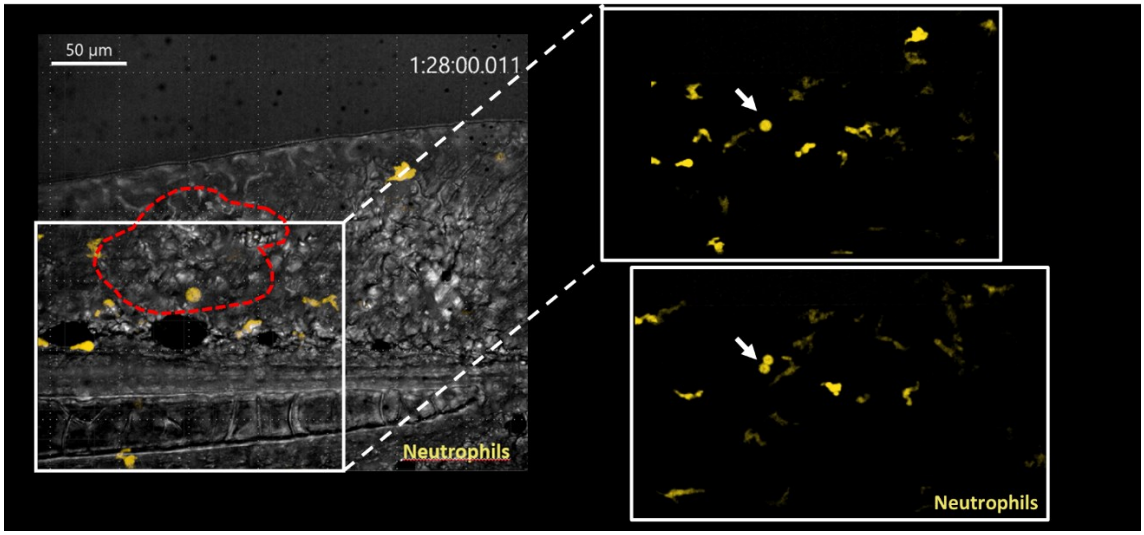

Figure 54. Neutrophil division in the chronically inflamed skin of Spint1a-deficient larvae. Screenshot showing a neutrophil division (arrow) in the vicinity of a keratinocyte aggregate (dotted red line).

The next aspect of neutrophil behavior that we wanted to analyze was neutrophil reverse migration. Neutrophils reverse migration has been deeply studied in zebrafish models of acute inflammation. In acute inflammation, like a sterile wound, neutrophils migrate from the CHT to the inflamed area, reaching the peak of neutrophils recruited at 5-6 post-wounding. After this time, neutrophils start leaving the inflamed area promoting the resolution of the inflammation in a process called reverse migration (Q. Deng & Huttenlocher, 2012; Ellett, Elks, Robertson, Ogryzko, & Renshaw, 2015; Shelef, Tauzin, & Huttenlocher, 2013; Starnes & Huttenlocher, 2012). We used *Tg(lyz:Dendra2)* after knocking down *spint1a* with CRISPR/Cas9 technology. In this transgenic zebrafish line, neutrophils accumulate Dendra2, a green fluorescent protein that, after excitation with a 405 nm laser turns its color into red (A. Y. Hsu et al., 2017; A. Y. Hsu et al., 2019). In this model, we partially photoconverted skin neutrophils from green to red at 2 dpf and they were visualized at 16 h post-photoconversion in yellow (**Figure 55A**). The results showed increased number of non-photoconverted neutrophils in the skin (green neutrophils) and decreased number of neutrophils photoconverted in the skin (yellow neutrophils). Additionally, non-photoconverted neutrophils were observed in other tissues, including muscle and the CHT (**Figure 55B-D**).

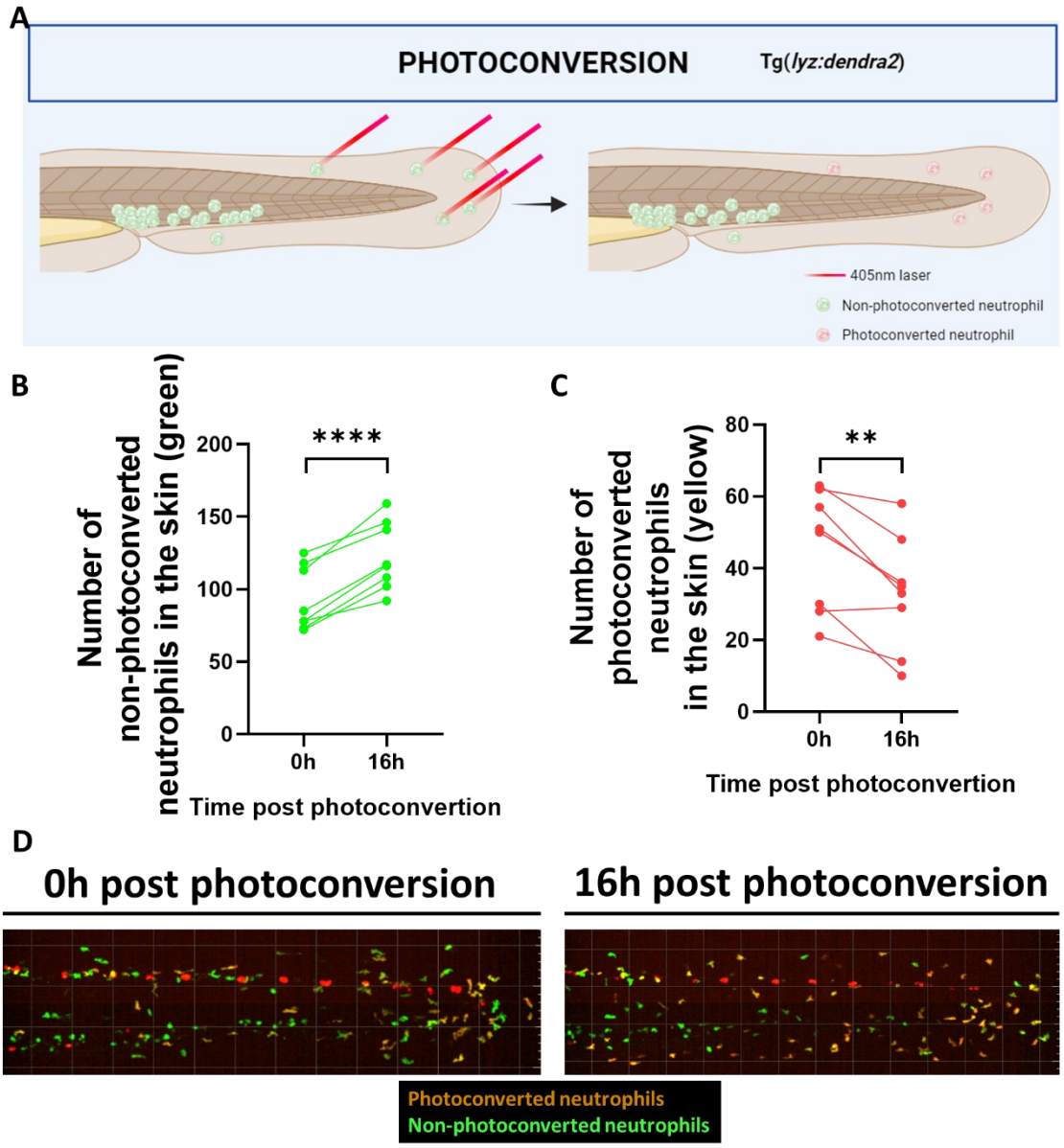

Figure 55. Neutrophil photoconversion in Spint1a-deficient mutant larvae. (A) At 2 dpf, *Tg(lyz:dendra2)* zebrafish neutrophils were partially photoconverted using a 405 nm laser (green plus red) and imaged at 0 and 16 h post-photoconversion. **(B, C)** Quantification of non-photoconverted (B) and photoconverted (C) neutrophils in the skin of the larvae at 0 and 16 h post-photoconversion. **(D)** Representative merge images showing photoconverted (yellow) and non-photoconverted (green) neutrophils. Each dot represents one individual whose neutrophils have been quantified at 0 and 16 h post-photoconversion. p-Values were calculated using paired t test. **p ≤ 0.01, ****p ≤ 0.0001.

The relevance of neutrophils in the exacerbation of inflammation and their behaviors prompted us to study whether Gapdh nuclear translocation played a role in neutrophil pro-inflammatory activity and behavior using pharmacological and genetic inhibition approaches. Pharmacological inhibition of Gapdh nuclear translocation with CGP3466B resulted in reduced neutrophil dispersion and specifically neutrophils recruited into the skin (**Figure 56A-C**). In addition, it was observed that the number of neutrophils interacting with keratinocyte aggregates was drastically

reduced by CGP3466B, while oxamate, which inhibits lactate dehydrogenase (LDH) and alleviated skin inflammation in this model (Naranjo-Sánchez, 2023), reduced the number of keratinocyte aggregates and neutrophil infiltration into the skin, but failed to reduce the number of neutrophils-keratinocyte aggregate interactions (**Figure 56D-E**). Similarly, CGP3466B was also able to reduce the number of keratinocyte aggregates in the Spint1a-deficient larvae upon Csf3a-induced neutrophilia (**Figure 57A, B**), despite the increased number of skin infiltrated neutrophils promoted by Csf3a (**Figure 57A, C**). Moreover, CGP3466B was able to decrease the total number of neutrophils in both cases (**Figure 57D**).

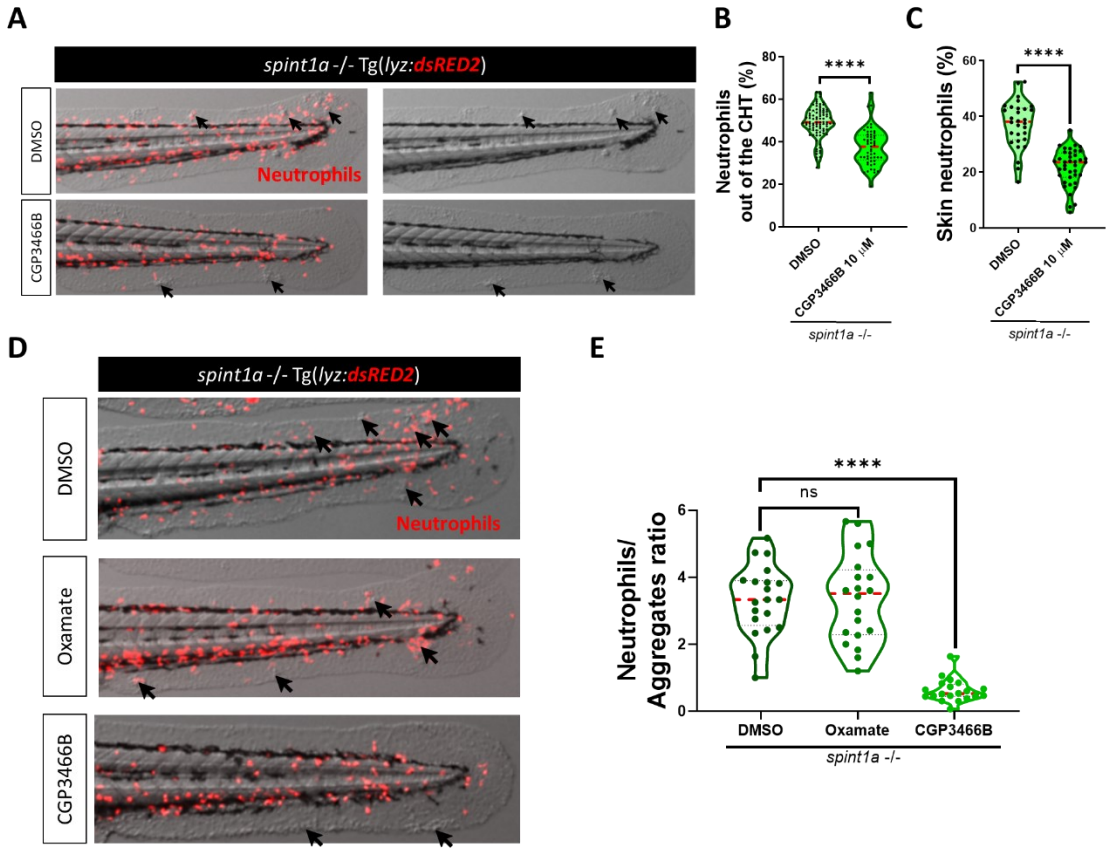

Figure 56. Pharmacological inhibition of Gapdh nuclear translocation impairs neutrophil skin recruitment and their interactions with keratinocyte aggregates in the inflamed skin. (A) Representative merge images showing skin and neutrophils in Spint1a-deficient larvae treated with either DMSO or 10 µM CGP3466B. Black arrows indicate keratinocyte aggregates. **(B)** Percentage of neutrophils out of the CHT. **(C)** Percentage of neutrophils recruited to the skin. **(D)** Representative merge images showing the skin and neutrophils of Spint1a-deficient larvae treated with either DMSO, Compound O or CGP3466B. Black arrows indicate the keratinocyte aggregates. **(E)** Quatitation of neutrophil/keratinocyte aggregate interactions. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using *t* test and 1-way ANOVA followed by Tukey multiple range test. ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; CHT, caudal hematopoietic tissue; ANOVA, analysis of variance.

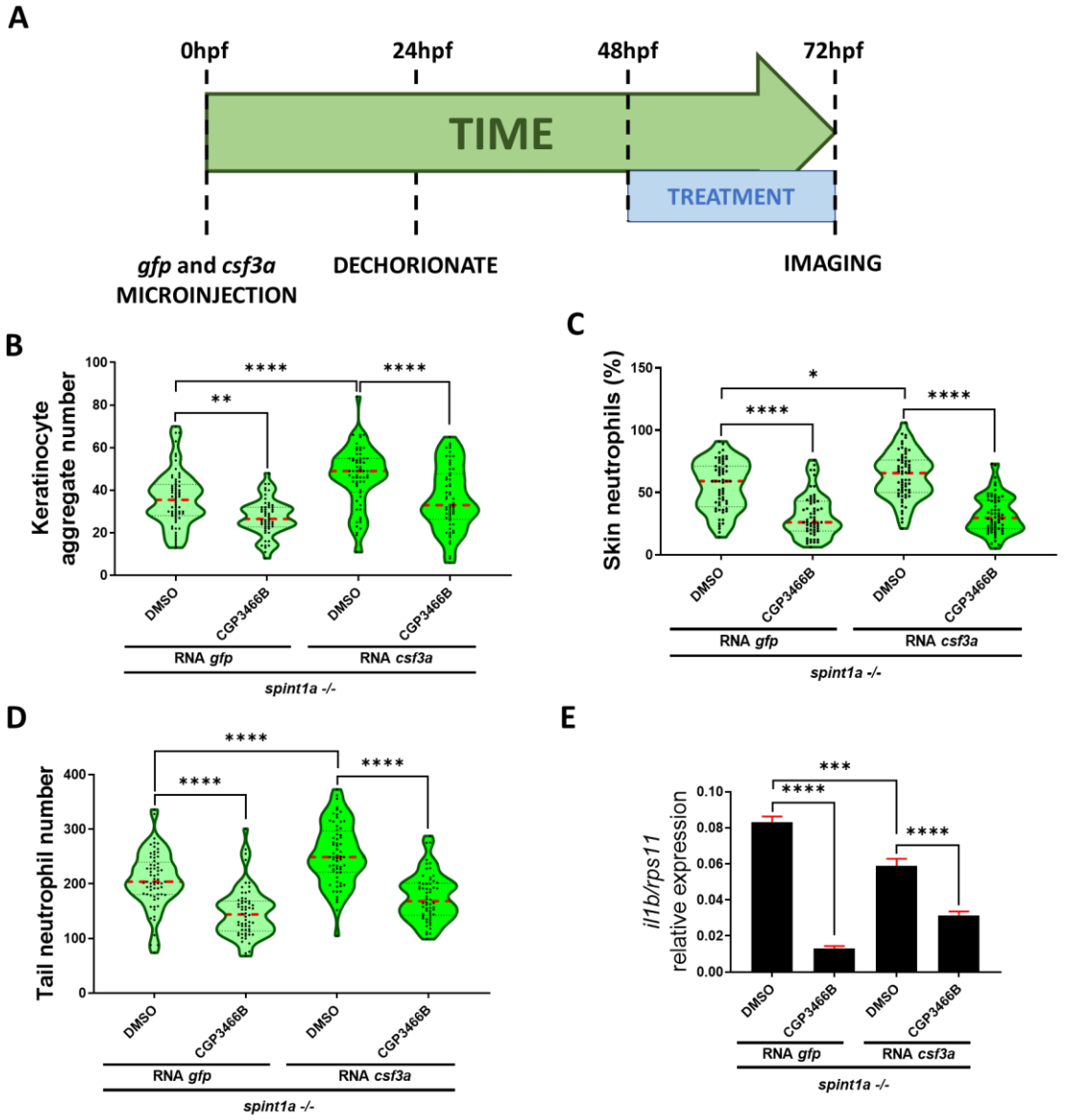

Figure 57. Pharmacological inhibition of Gapdh nuclear translocation impairs neutrophil skin recruitment in the inflamed skin upon Csf3a-induced neutrophilia. (A) Spint1a-deficient zebrafish embryos were microinjected in one-cell stage with *csf3a* and *gfp* mRNAs, dechorionated at 24 hpf, treated with either DMSO or 10µM CGP3466B at 48 hpf, and imaged and analyzed at 72 hpf. **(B)** Number of keratinocyte aggregates in *gfp* and *csf3a*-microinjected Spint1a-deficient larvae. **(C)** Neutrophil recruitment to the skin. **(D)** Total number of neutrophils. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA followed by Tukey multiple range test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

We next analyzed the impact of CGP3466B at different time points in order to clarify if pharmacological inhibition of Gapdh nuclear translocation was able to impair keratinocyte aggregate formation, leading to reduced neutrophil recruitment or, alternatively, if directly reduced neutrophil recruitment, impairing neutrophil-mediated exacerbation of the phenotype

(**Figure 58A**). As a control, the NAMPT inhibitor FK-866, whose treatment has previously been reported to rescue chronic skin inflammation in the Spint1a-deficient model (Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021), was used. At 48 hpf, after 24h of treatment, the number of keratinocyte aggregates was not affected by CGP3466B, but FK-866 reduced them as soon as 48 hpf (**Figure 58B**). Nevertheless, at 72hpf, FK-866 also reduced keratinocyte aggregate number, while CGP3466B treatment slightly reduced them (Fig. 58B). As regards neutrophil recruitment to the skin, CGP3466B drastically reduced it at 48 hpf, even was mor powerful than FK-866, while both treatments reduced the neutrophil recruitment to the skin at 72 hpf (**Figure 58C**). Finally, although both treatments reduced the total number of neutrophils at 72 hpf, only CGP3466B was able to do so at 48 hpf (**Figure 58D**).

Although the above results indirectly suggest that pharmacological inhibition of Gapdh nuclear translocation impaired neutrophil recruitment to inflamed skin, an effect of this non-canonical function of Gapdh cannot rule out. Therefore, we performed rescued experiments using with plasmids encoding zebrafish wild type Gapdh and the nuclear translocation defective mutant Gadph-C152S driven by the neutrophil specific promoter *lyz*. The plasmids used also harbored a *cry:ECFP* transgene which allows expression of *ECFP* in the crystallin of the microinjected zebrafish larvae for rapid screening (**Figure 59A-B**). Zebrafish *spint1a-/-* embryos were microinjected with either plasmid, and imaged and analyzed at 72hpf. Forced expression of wild type Gapdh in neutrophils increased the number of keratinocyte aggregates, while the mutant Gapdh-C150S failed to do so (**Figure 59C-D**). Similar results were obtained regarding neutrophil recruitment into the skin (**Figure 59C and E**). However, the total number of neutrophils was not altered by the neutrophil-specific expression of either Gapdh (**Figure 59C, F**). By RT-qPCR, we confirmed similar mRNA levels of both *gapdh* (**Figure 59G**).

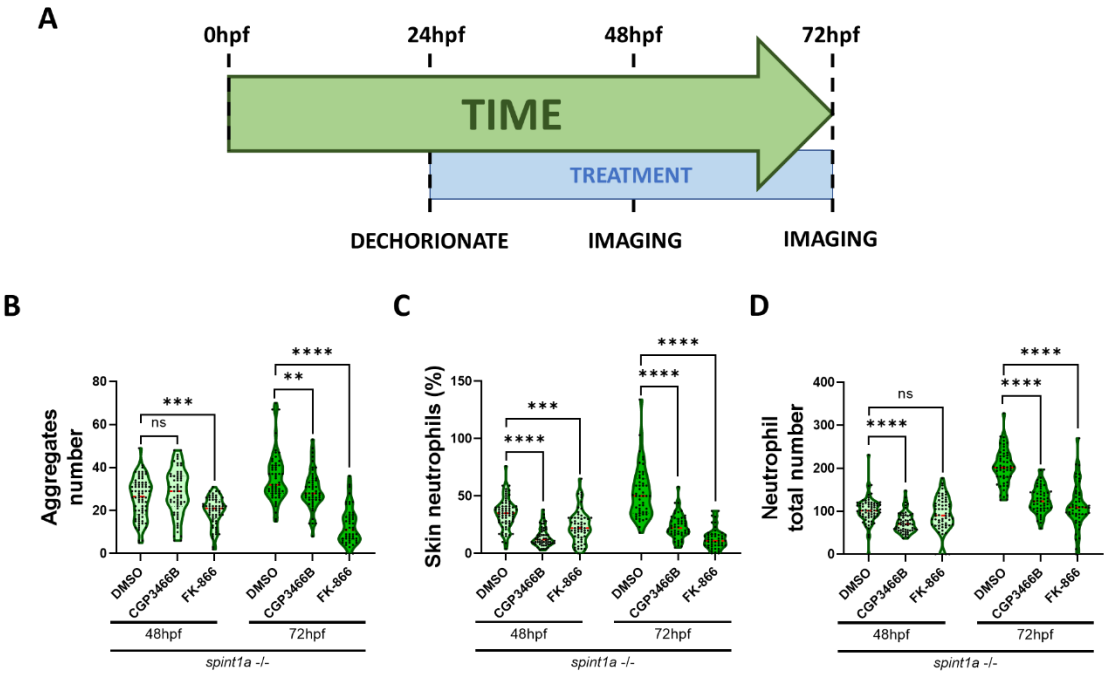

Figure 58. Dynamic effects of pharmacological inhibition of Gapdh nuclear translocation and NAMPT in chronic skin inflammation. (A) Spint1a-deficient larvae were treated from 24 hpf with the inhibitors CGP3466B and FK-866, and imaged and analyzed at 48 and 72hpf. **(B)** Keratinocyte aggregates number. **(C)** Percentage of neutrophils recruited to the skin. **(D)** Total number of neutrophils. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA followed by Tukey multiple range test. Ns, no significance, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; CHT, caudal hematopoietic tissue; ANOVA, analysis of variance; hpf, hours post fertilization.

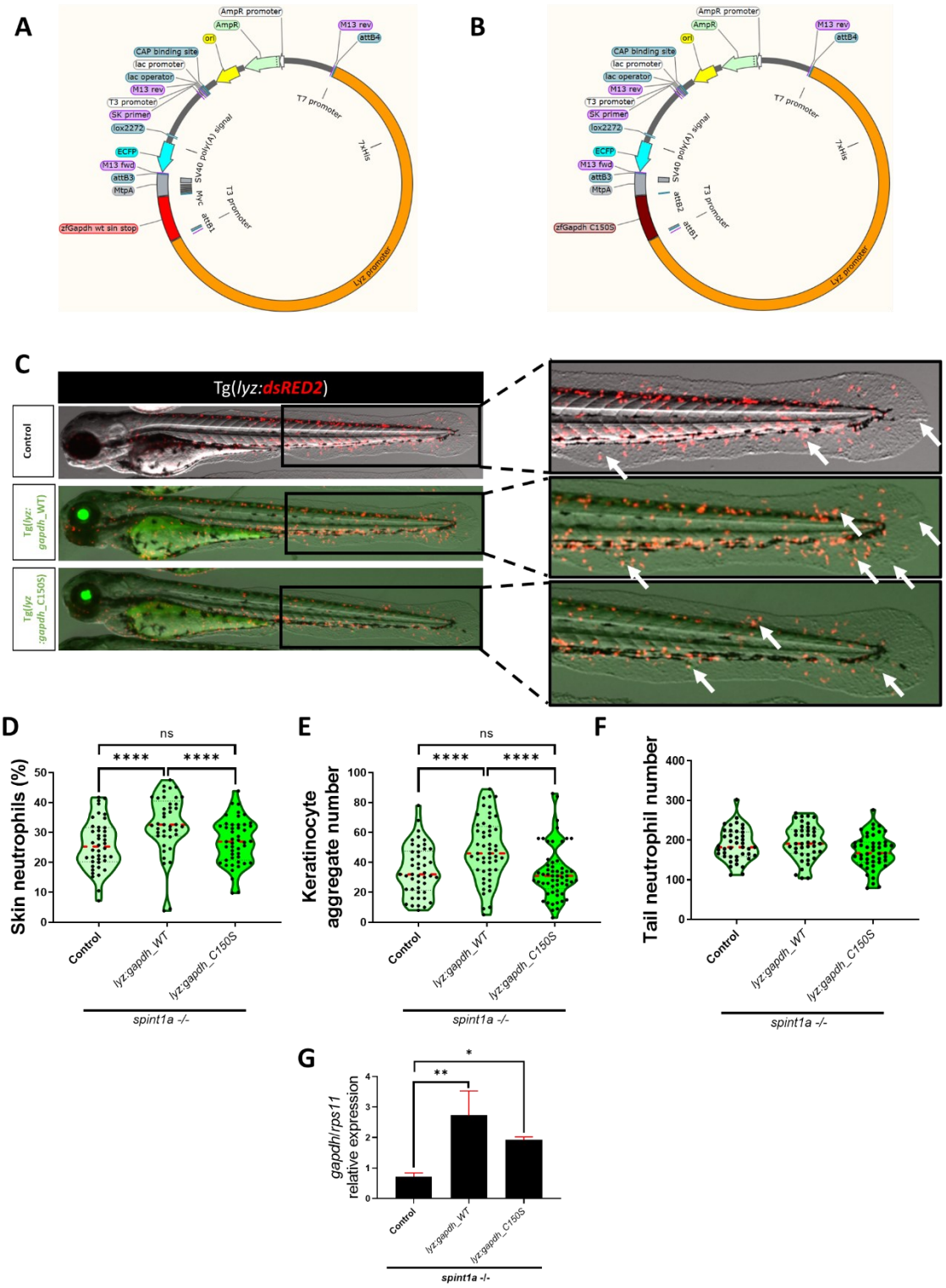

Figure 59. Gapdh nuclear translocation regulates in a cell-autonomous manner neutrophil recruitment to the inflamed skin. (A) Map of plasmid *Tg(lyz:gapdh)*. The plasmid encodes wild type Gapdh driven by the *lysozyme* promoter and also contains the reporter *cry:ECFP*. **(B)** Map of plasmid *Tg(lyz:gapdh^{C150S}).* The plasmid nuclear translocation defective Gapdh-C150S driven by the lysozyme promoter and the reporter *cry:ECFP*. **(C)** Representative merge images showing skin, neutrophils, and crystallin of Spint1a-deficient larvae non-microinjected (control), and microinjected with *Tg(lyz:gapdh) or Tg(lyz:gapdh^{C150S})* plasmids. White arrows label the keratinocyte aggregates in the skin. **(D)** Percentage of neutrophils in the skin. **(E)** Number of keratinocyte aggregates. **(F)** Total number of neutrophils. **(G)** RT-qPCR analysis of *gapdh* mRNA levels. Each dot represents one individual, and the median for each group is also shown. p-Values were

calculated using 1-way ANOVA followed by Tukey multiple range test. Ns, no significance, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ANOVA, analysis of variance; *lyz, lysozyme;* WT, wildtype*; rps11, ribosomal protein S11; gapdh, glyceraldehyde 3-phosphate dehydrogenase.*

The above results encourage us to determine the effect of Gapdh nuclear translocation in the neutrophil behavior in the inflamed skin using *Tg(lyz:BFP)* neutrophils, which express the reporter BFP protein specifically in neutrophils (Rosowski et al., 2018). It was observed that those neutrophils which were interacting with the keratinocyte aggregates stayed in the same place during longer times than the neutrophils which interacted with other keratinocytes out of the aggregates. Thus, some neutrophils were interacting with the different aggregates observed in the skin for at least 2 h (**Figure 60A-C**). Strikingly, pharmacological inhibition of Gapdh nuclear translocation with CGP3466B resulted in reduced time of interaction, reaching the levels of neutrophils interacting with keratinocytes out of the aggregates. The speed of the neutrophils was also measured in the generated movies (**Supplementary videos 2 and 3**), showing a significant reduction after the treatment with CGP3466B (**Figure 60D**). These results were further confirmed by genetic inhibition of *gapdh* with CRISPR/Cas9. Thus, genetic inhibition of *gapdh* also decreased the interaction time between neutrophils and keratinocyte aggregates (**Figure 61 and Supplementary videos 4 and 5**).

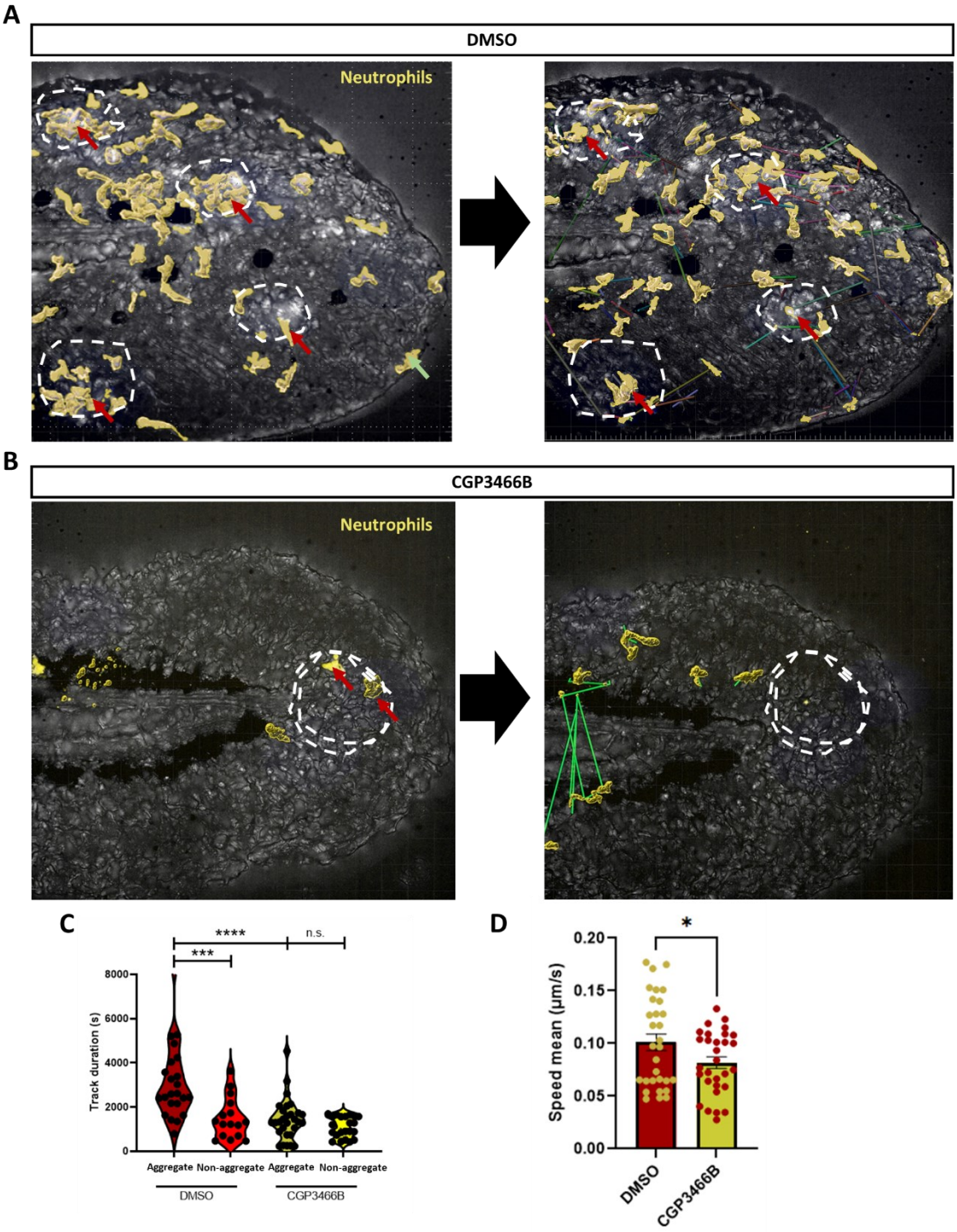

Figure 60. Neutrophil-keratinocyte interactions in the inflamed skin of Spint1a-deficient larvae is modulated by Gapdh nuclear translocation. *Tg(lyz:BFP)* were microinjected with *spint1a* crRNA/Cas9 complexes and recorded during 4h at 72 hpf using an spinning disk confocal microscope. **(A, B)** Representative merge images of long-term movies of larvae treated with either DMSO (A) or CGP3466B (B). Left images were taken at 0 h and right images at 2 h. The keratinocyte aggregate areas are depicted with a white dotted line, while red arrows label the neutrophils interacting with them. The neutrophils are colored in yellow with their tracks represented in dragon tail format. **(C)** The mean time each neutrophil was interacting with the keratinocytes was measured. **(D)** Mean speed of each neutrophil. Each dot represents the mean of all the neutrophils of one individual during the 4 h of the movie, and the median and mean ± SD for each group is also shown. p-Values were calculated using 1-way ANOVA followed by

Tukey multiple range test and t test. Ns, no significance, *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001. ANOVA, analysis of variance; lyz, lysozyme; DMSO, dimethyl sulfoxide; s, seconds; µm, micrometers.

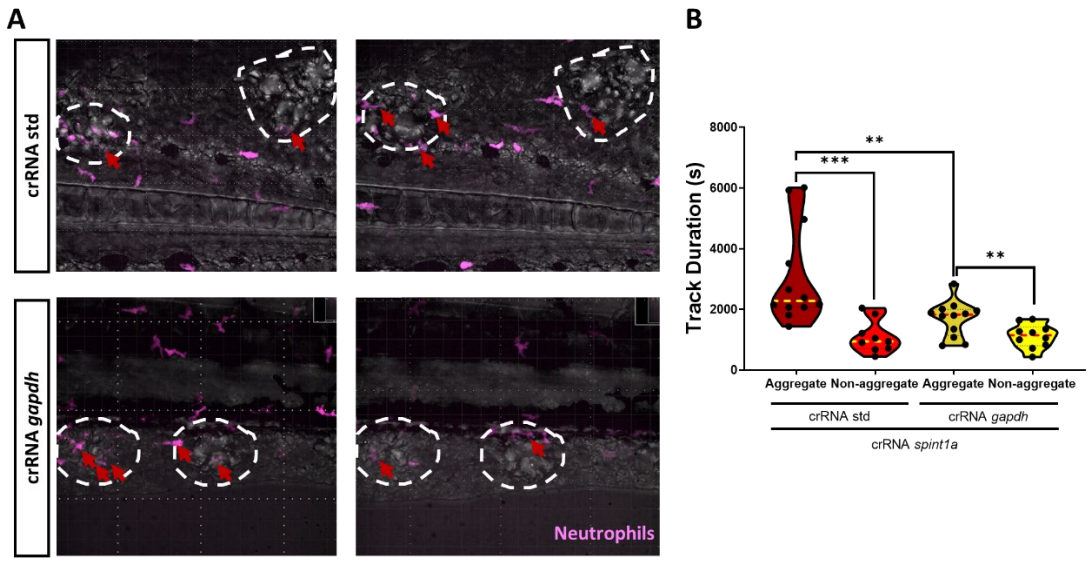

Figure 61. Genetic inhibition of Gapdh reduces the interaction between neutrophil and keratinocyte aggregated of the inflamed skin of Spint1a-deficient larvae. *Tg(lyz:BFP)* eggs were microinjected with *spint1a* crRNA alone or combined with *gapdh* crRNA in the presence of recombinant Cas9. **(A)** Representative merge images of long-term movies of 72 hpf larvae obtained with a spinning disk confocal microscope. Left images were taken at 0 h and right images 2h later. Aggregate areas are depicted with white dotted lines, and red arrows label the neutrophils (pink) interacting with the keratinocyte aggregates. **(B)** Mean time each neutrophil was interacting with the keratinocytes. Each dot represents the mean of all the neutrophils of one individual during the 4h of the movie, and the median for each group is also shown. p-Values were calculated using 1-way ANOVA followed by Tukey multiple range test. **p ≤ 0.01, ***p ≤ 0.001. ANOVA, analysis of variance; *lyz, lysozyme*; crRNA, CRISPR RNA; s, seconds; std, standard; *gapdh, glyceraldehyde 3-phosphate dehydrogenase*.

· Discussion

The main objective of this chapter was to understand the role of the neutrophils in the progression of the chronic skin inflammation which characterizes the Spint1a-mutant larval model, and then correlate Gapdh nuclear translocation with neutrophil activity. We found that the number of neutrophils infiltrated in the skin of Spint1a-deficient larvae positively correlated with skin inflammation and, more importantly, manipulation of neutrophil number using two different experimental approaches affected skin inflammation. This result contrasts the inability of neutrophil ablation using a morpholino against the gene encoding the master myeloid transcription factor Spi1 (also known as pu.1) to reduce keratinocyte aggregates in this model (PMID: 17728346). The discrepancy could be explained by the different experimental approaches used, since genetic inhibition of *spi1* would also result in macrophage ablation and the role of this cell type in the skin inflammation of this model is unknown. However, both studies demonstrate that neutrophils are not causative of the epidermal defects but rather exacerbated it.

Another interesting observation is that neutrophils are able to divide in the skin. This process has been observed to take place near the keratinocyte aggregates, where neutrophils stop, acquire a spherical shape, then divide producing two different new neutrophils that continue migrating through the skin. This unexpected process was previously observed by Huttenlocher and collaborators in this model and they speculated that dividing cells were probably neutrophil precursors that also express *mpx* (PMID: 17881499). Our scRNA-seq confirmed this hypothesis, since we identified a subcluster of neutrophils (number 6), which is not present in wild type larvae, and expressed 35 genes involved in cell cycle progression and proliferation, but did not express HSPC markers. As we have discussed in the previous Chapter, this cluster might represent neutrophils generated by emergency granulopoiesis (Gullotta et al., 2023; A. J. Kwok et al., 2023; I. Kwok et al., 2020; Manz & Boettcher, 2014). As immature neutrophils have been associated to the development of some diseases (Gullotta et al., 2023; A. J. Kwok et al., 2023; I. Kwok et al., 2020; Manz & Boettcher, 2014), the Spint1a-deficient model may be used to study emergency myelopoiesis and the relevance of immature neutrophils in chronic inflammatory diseases. The genes found to be enriched in this neutrophil subcluster may be used to generate appropriate transgenic reporter lines to track immature neutrophils *in vivo* and to ablate them to determine their contribution to the inflammatory phenotype of this model. Furthermore, it would be interesting to determine the inflammatory signals involved in the recruitment of immature neutrophils and whether the keratinocyte aggregates release specific factors that promote cell division, “replacing” the niche of the CHT. Conversely, our results also suggests that neutrophils

may release factor that trigger exacerbate keratinocyte aggregate proliferation and skin inflammation. For example, it would be interesting to check if recruited neutrophils carry out other functions, like NETosis, which was recently reported to take place in psoriasis triggered by keratinocyte exosomes release (M. Jiang et al., 2019). The identification of specific factors, i.e. chemokines and DAMPs, enriched in the specific keratinocyte populations of the Spint1a-deficient larvae obtained in the scRNA-seq analysis, in combination with the identification of the receptors expressed by the specific populations of neutrophils might be an insightful approach.

Neutrophil reverse migration is an important mechanism to trigger inflammation resolution of neutrophils in the inflamed or damaged tissues, in which they leave the injured site and migrate back to vasculature, in order to avoid the possible negative effects of neutrophils after swarming (Isles et al., 2021; Kienle et al., 2021; Mihlan et al., 2022; B. Rocha-Gregg & A. Huttenlocher, 2021). Our photoconversion study in the Spint1a-deficient model showed a rapid turnover of neutrophils in the inflamed skin where "naïve" neutrophils probably migrating from the CHT to the skin rapidly replace previously recruited neutrophils. A relevant question is the fate of neutrophils upon their recruitment to the skin and their long-lasting interaction with keratinocyte aggregates because we did not find any evidence of reverse migration from the skin to the CHT. Unfortunately, due to technical difficulties, we were unable to analyze other distant tissues but, as we did not find any evidence of neutrophil death, it is tempting to speculate that neutrophils disseminated to other tissues and spread inflammation. This is not surprising, since it has been reported in both mice and zebrafish that neutrophils recruited to the inflamed foci, called wound-sensitized neutrophils, can reverse migrate to other no-inflamed tissues without losing their activated state and then disseminating the inflammation systemically, and even leading to comorbidities associated to chronic inflammatory diseases (Barkaway et al., 2021) (PMID: 21248150, PMID: 21532050).

One of the most interesting observations of this Chapter is that both pharmacological inhibition of Gapdh nuclear translocation and genetic inhibition of the specific Gapdh isoform of neutrophils decreased the percentage of neutrophils infiltrated in the skin and reduced their speed and duration of neutrophil-keratinocyte aggregate interactions. Furthermore, the LDH inhibitor oxamate, which reduced the number of keratinocyte aggregates and neutrophil infiltration into the skin, surprisingly, failed to reduce the number of interactions between neutrophils and keratinocyte aggregates. Similarly, pharmacological inhibition of Gapdh nuclear translocation failed to reduce the number of keratinocyte aggregates, but not neutrophil infiltration into the skin, at an earlier time point (24 hpf), while inhibition of Nampt with FK-866, which directly reduces keratinocyte hyperproliferation and cell death (PMID: 34748530), was

able to reduce both neutrophil infiltration and the number of keratinocyte aggregates at this early time point. Although all these results taken together suggest that the nuclear translocation of Gapdh in neutrophils regulates their interaction with the keratinocyte aggregates of the inflamed skin, rescued experiments performed in Gapdh-deficient larvae by forced expression of wild type and nuclear translocation defective Gapdh-C152S unequivocally demonstrated a cell-autonomous effect of non-canonical function of Gapdh in neutrophil migration, activation and exacerbation of skin inflammation in Spint1a-deficient larvae.

It is complicated to envisage the molecular mechanism orchestrating neutrophil behavior and pro-inflammatory activity by nuclear Gapdh because the available information on the non-canonical functions of Gapdh in neutrophils is rather limited. NETosis is one of the few examples reported to be regulated by GAPDH, but in this case by its canonical function, and considering its correlation with the severity of psoriasis in patients and its involvement on activation and cytokine release by keratinocytes (Awasthi et al., 2023; M. Jiang et al., 2019; B. Matta et al., 2022), it would be worthy to study whether Gapdh regulates NETosis in the Spint1a-deficient model using available reporter lines, such as *Tg(lyz:H2B-mCherry)* (S. K. Yoo et al., 2012), and the possible relevance of this process in the exacerbation of skin inflammation mediated by neutrophils.

In summary, it has been shown in this Chapter that neutrophils played a negative role in the progression of chronic skin inflammation with a robust correlation of the number of infiltrated neutrophil and the number of keratinocyte aggregates of the *spint1a-/-* model. Moreover, it has been shown that Gapdh nuclear translocation was involved in neutrophil behavior during chronic skin inflammation, since its inhibition resulted in reduced neutrophil migration and speed, and in shorter interaction with keratinocyte aggregates of the inflamed skin. The next objective will focus on the understanding of the mechanism involved in the regulation of neutrophils activation by the nuclear translocation of Gapdh.

Chapter 4: Gapdh nuclear translocation modulates *il4* expression in neutrophils during chronic skin inflammation

· Results

In order to understand the mechanism regulated by Gapdh nuclear translocation in neutrophils, in a chronic skin inflammation context, Spint1a-deficient larval tail treated with CGP3466B from 48 to 72 hpf was also analyzed by single cell RNA-seq. The clustering of the different cells included in the analysis provided a total of 17 clusters: neurons, glia cells, muscle cells, neutrophils, median fin fold, endothelial cells, myeloid precursors, basal keratinocytes, peridermal keratinocytes, erythrocytes, fibroblasts, neuromasts, mesoderm, neuron precursors, notochord, neural crest and macrophages (**Figure 62A**). The different markers used to annotate the clusters obtained are shown in the heatmap represented in **Figure 62B**.

We focused our attention on neutrophils in order to characterize the population and find some features of the neutrophils in inflammatory conditions and upon treatment with the inhibitor of Gapdh nuclear translocation. For this, the neutrophil cluster, characterized by the expression of *lyz, mmp13a, npsn* and *mpx* (**Figure 62B**), was selected and studied (**Figure 63A**). We found that inhbtion of Gadh nuclear translocation reduced the transcript levels of keratin 4 (*krt4*), *col1a1a*, *col1a2*, dual specificity phosphatase 5 (*dusp5*) and interleukin 4 (*il4*), while increasing those of pleckstrin homology domain containing, family F (with FYVE domain) member 1 (*plekhf1*), transcobalamin beta b (*tcnbb*), NF-κB inhibitor A (*nfkbiaa*) and *pim2* (**Figure 63B, C & D**).

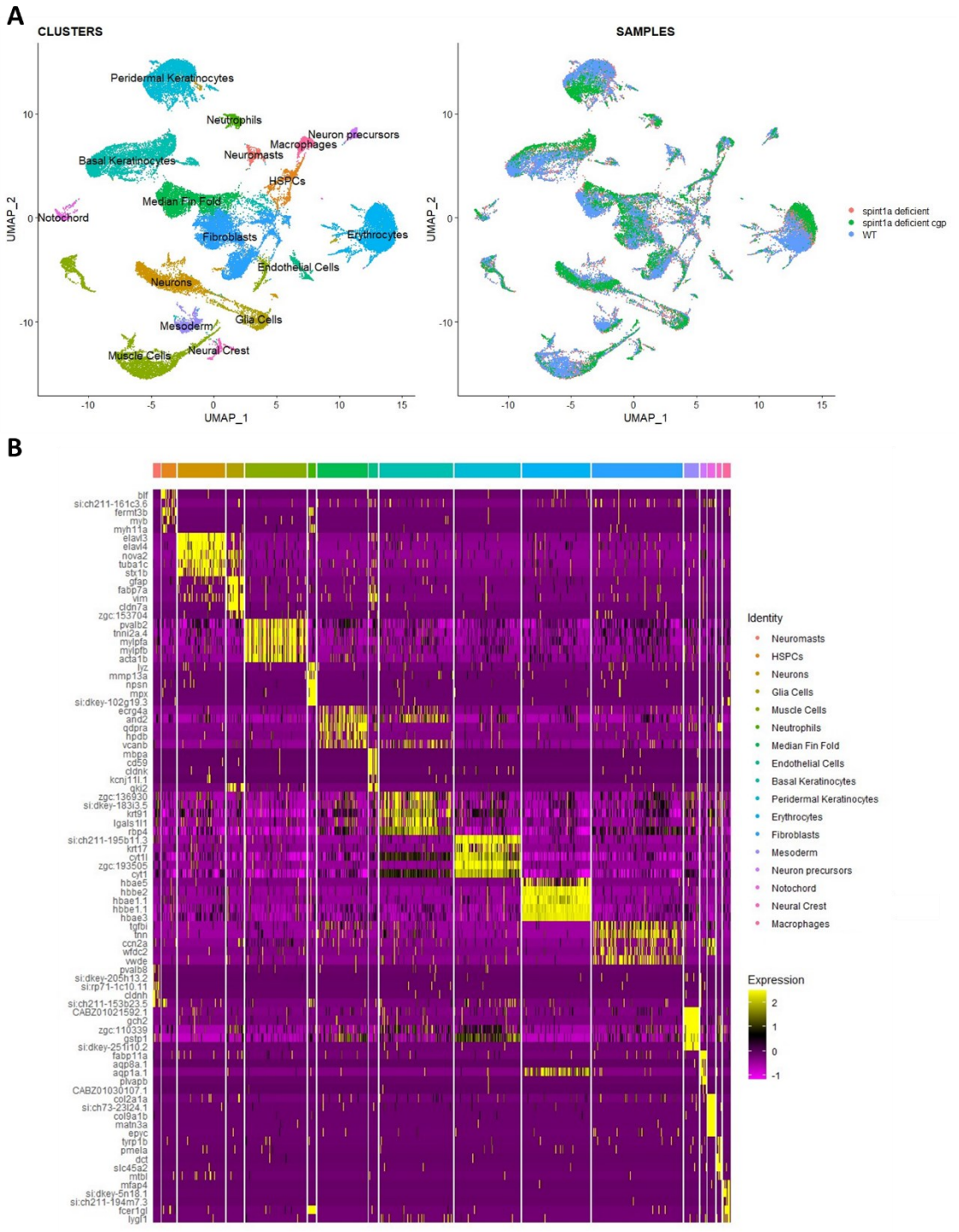

Figure 62. scRNA-seq data of larval tails from wildtype and Spint1a-deficient larvae untreated or treated with CGP3466B. (A) UMAP representing the different clusters (left) and the different sample origin (right) of the cells analyzed. The filtering and clustering of the cells provided a total of 17 different clusters. **(B)** Heatmap showing the five most representative markers of each annotated cluster. The list was obtained with the FindAllMarkers function in Seurat package, determined using Wilcoxon Rank Sum test to identify differentially expressed genes between groups. UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

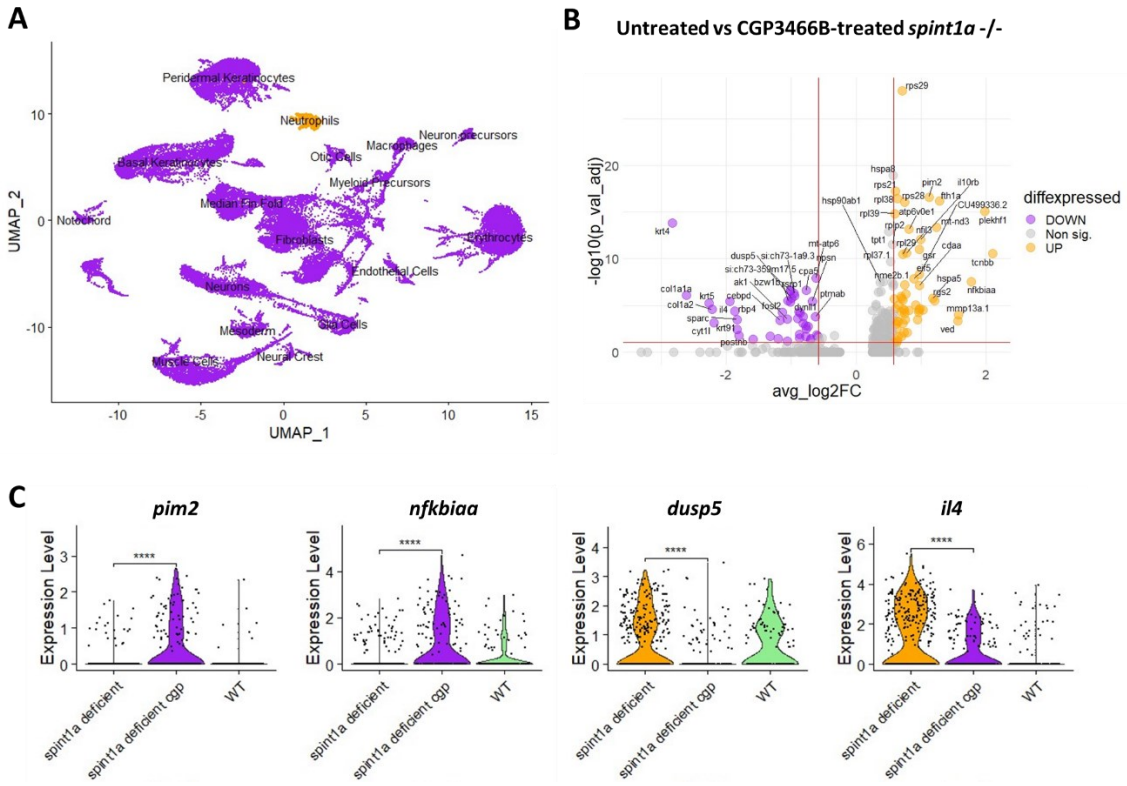

Figure 63. scRNA-seq of tail neutrophils from Spint1a-deficient larvae untreated or treated with CGP3466B. (A) UMAP representing the different clusters in the scRNA-seq, highlighting in orange the neutrophil population. **(B)** Volcano plot resulting from the gene expression comparison between untreated and treated with CGP3466B Spint1a-deficient neutrophils. In orange, upregulated genes in Spint1a-deficient; in purple, downregulated genes in Spint1a-deficient compared to Spint1a-deficient treated with CGP3466B. (C) Violin Plot representing the differential expression of some representative genes in the differential expression analysis. The genes *pim2* and *nfkbiaa* were upregulated after the treatment with CGP3466B, while the genes *dusp5* and *il4* were downregulated. Each point represents the gene expression level in a different cell analyzed. p-Values were calculated using Wilcoxon Rank Sum test. ****p ≤ 0.0001. UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction.

With the aim of analyzing in more detail the neutrophil population, the neutrophil cluster was selected and reclustered individually, obtaining a total of nine clusters, numbered from 0 to 8, composed by neutrophils from the three different samples, WT, Spint1a-deficient and Spint1a-deficient treated with CGP3466B (**Figure 64A**). The different neutrophil subclusters were classified depending on each particular gene expression pattern. Thus, the three most differentially expressed genes of each cluster are represented in the **Figure 64B**. All the clusters were composed by cells of the three samples analyzed together, except in the cluster 3, where there are no WT neutrophils. The WT neutrophils were also barely present in the clusters 1 and 4. Moreover, the cluster 3 was mainly formed by neutrophils from CGP3466B-treated larvae. The untreated Spint1a-deficient neutrophils were the most prevalent in almost every cluster, except clusters 2 and 3, but interestingly, the cluster 1 was mainly formed by only these neutrophils (**Figure 64C**). Looking at this neutrophil subcluster organization, the cluster 3, specifically formed

by neutrophils of the *spint1a* -/- larvae treated with Gapdh nuclear translocation inhibitor, emerged as an interesting population to study. Strikingly, the cluster was characterized by the expression of different genes related to inflammation, like tumor necrosis factor beta (*tnfb*), interleukin 10 receptor beta (*il10rb*) or NF-κB inhibitor A (*nfkbiaa*). The most differentially expressed gene in this cluster was the pleckstrin homology domain containing, family F (with FYVE domain) member 1 (*plekhf1*) (**Figure 64D**).

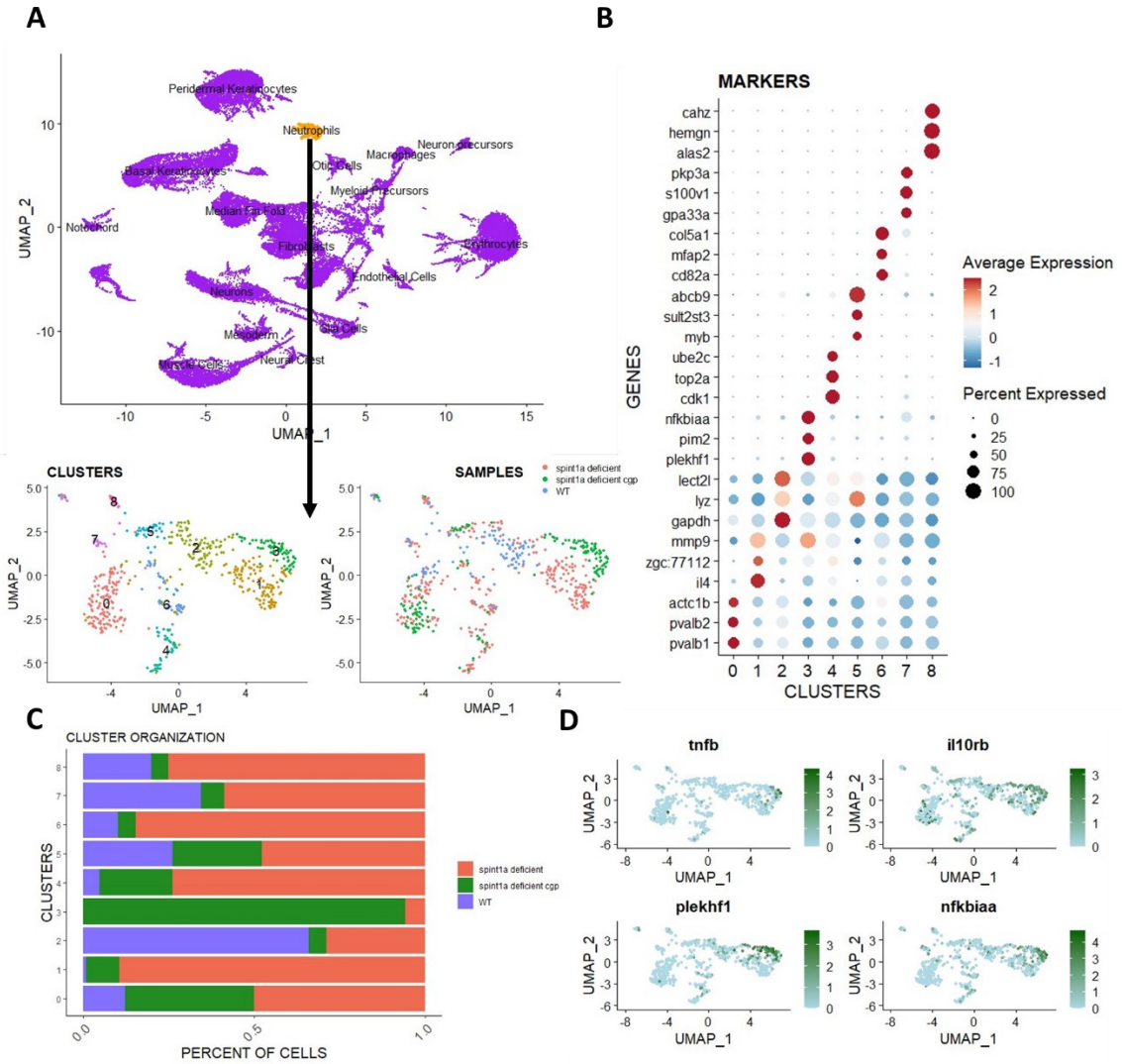

Figure 64. Neutrophil scRNA-seq subclustering and analysis. (A) UMAP showing the different clusters in the scRNA-seq, highlighting in orange the neutrophil cluster, which was subjected to reclustering. The reclustering provided in different clusters represented in the second UMAP. Left, neutrophils classified by clusters; right, neutrophils classified by sample origin. **(B)** Dotplot representing the three first markers of each neutrophil cluster, from 0 to 8. **(C)** Barplot showing the percentage of cells of each sample present on each cluster. **(D)** Feature plot showing the expression of the genes *tnfb*, *il10rb*, *plekhf1* and *nfkbiaa*, characteristic of the neutrophil cluster 3. WT, wildtype; UMAP, Uniform Manifold Approximation and Projection; *tnfb*, tumor necrosis factor beta; *il10rb*, interleukin 10 receptor beta; *plekhf1*, pleckstrin homology domain containing, family F (with FYVE domain) member 1; *nfkbiaa*, nfkb inhibitor alpha a.

Since the transcript levels of *nfkbiaa* increased in neutrophils of Spint1a-deficient larvae upon inhibition of Gapdh nuclear translocation, we wondered if the nuclear translocation of Gapdh was able to inhibit the activity of NF-κB, resulting in alleviation of inflammation in the *spint1a* -/- larvae. To check it, the reporter line *Tg(NF-κB:eGFP)* was used in order to measure the NF-κB-associated inflammation (**Figure 65A**). A significant decreased NF-κB signal was observed after the treatment with the inhibitor CGP3466B (**Figure 65A-B**). However, no correlation was observed between the number of neutrophils in the skin and NF-κB activity (**Figure 65C**). Moreover, genetic inhibition of *gapdh* or *gapdhs* failed to reduce NF-κB activity in the skin of *spint1a* -/- larvae (**Figure 65D**).

In order to check the functional relevance of the regulation of the NF-κB activity by Gapdh nuclear translocation, transgenic larvae expressing a dominant negative (DN) form of the NF-κB inhibitor Ikbaa, the protein encoded by the gene *nfkbiaa*, specifically in neutrophils were then used. To get that, *Tg(mpx:GAL4-VP16)* and *Tg(UAS:DN-Ikbaa)* were outcrossed, guaranteeing that all the descendance expressed the DN protein in the neutrophils (Robertson et al., 2014), and *spint1a* was inactivated by CRISPR-Cas9 technology. The results showed that inhibition of NF-kB activity in neutrophils failed to attenuate skin inflammation, assayed as the number of keratinocyte aggregated (**Figure 66A-B**), the number of neutrophils infiltrated into the skin (**Figure 66C**), the percentage of neutrophils out of the CHT (**Figure 66D**), and the total number of neutrophils (**Figure 66E**).

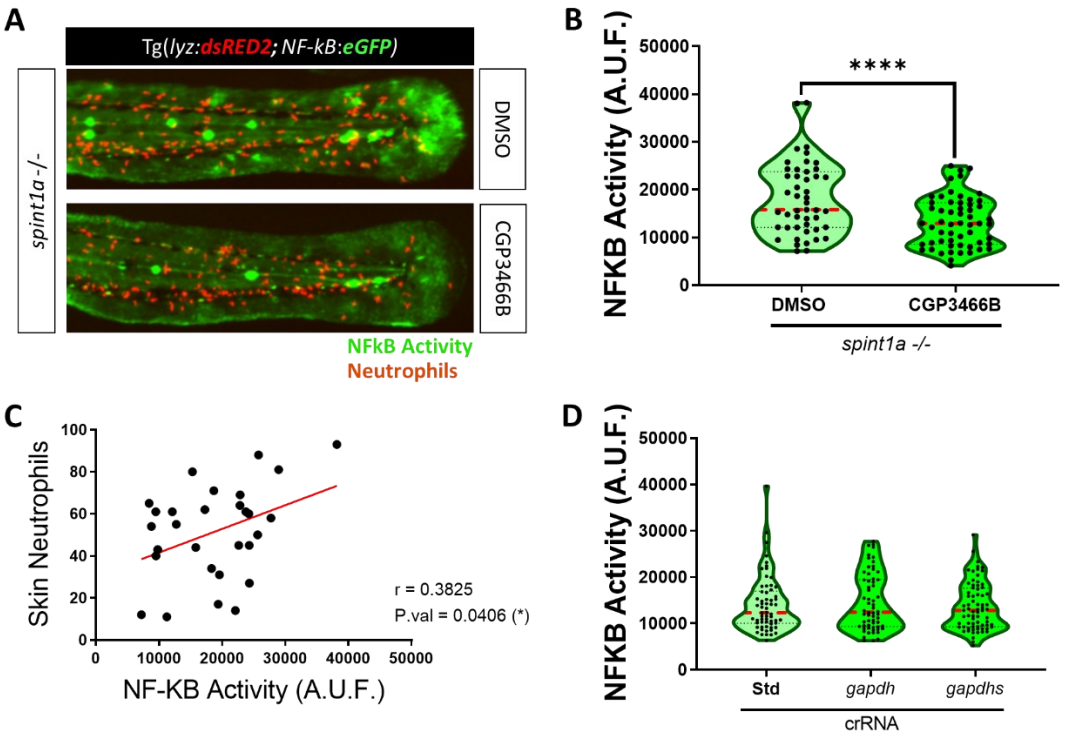

Figure 65. Effect of Gapdh nuclear translocation inhibition in NF-κB activity in Spint1a-deficient larvae. **(A)** Representative merged images of NF-κB activity and neutrophils of 3 dpf Spint1a-deficient larvae untreated (DMSO) and treated with 10 µM CGP3466B. **(B)** Violin plot showing NF-κB activity fluorescence intensity in untreated (DMSO) and treated with CGP3466B larvae. **(C)** Correlation between the number of skin neutrophils and NF-κB activity. **(D)** Violin plot showing NF-κB activity in the skin of Spint1a-deficient larvae after genetic inhibition of *gapdh* and *gapdhs*. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test and 1-way ANOVA followed by Tukey multiple range test. ****p ≤ 0.0001. DMSO, dimethyl sulfoxide; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *gapdhs*, glyceraldehyde 3-phosphate dehydrogenase, spermatogenic; A.U.F., Arbitrary Units of Fluorescence; crRNA, CRISPR RNA.

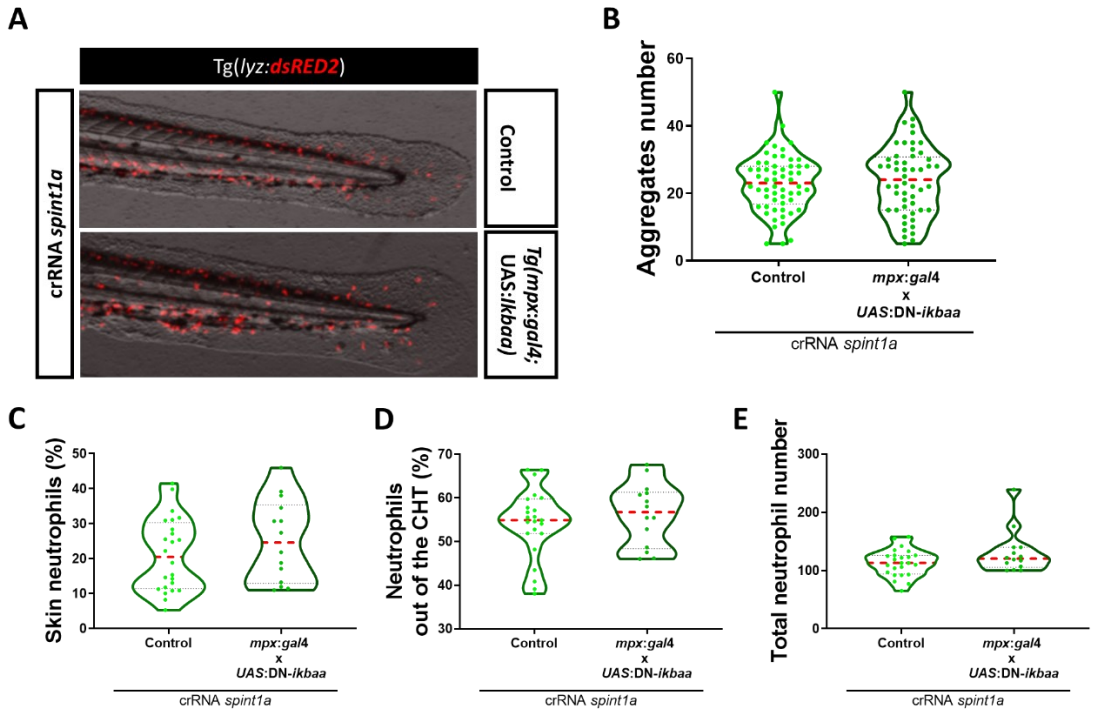

Figure 66. Effect of NF-κB inhibition in the *spint1a* -/- larvae. (A) Representative merged images of the skin and neutrophils of 3dpf WT (control) and *Tg(mpx:gal4;UAS:Ikbaa)* larvae upon genetic inhibition of *spint1a*. **(B)** Violin plot showing the number of keratinocyte aggregates. **(C)** Percentage of skin neutrophils. **(D)** Percentage of neutrophils out of the CHT. **(E)** Total number of neutrophils. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. crRNA; CRISPR RNA; CHT, Caudal Hematopoietic Tissue; DN; Dominant Negative; WT, wildtype.

We next wanted to know whether the effect of the inhibitor CGP3466B on the induction of some inflammatory genes was specific for neutrophils or it also occurred in other cells. To study that, the expression of the selected genes was analyzed in neutrophils and peridermal and basal keratinocytes (**Figure 67A**), as these 2 keratinocyte population are involved in skin inflammation. In peridermal keratinocytes, all the genes that were upregulated in neutrophils, except *il6r*, whose expression was low in keratinocytes, were upregulated as well (**Figure 67B-C**). In the case of the basal keratinocytes, all the genes upregulated in neutrophils were also upregulated, with the exception of *cxcr3.2* and *cxcl19* (**Figure 67B-D**).

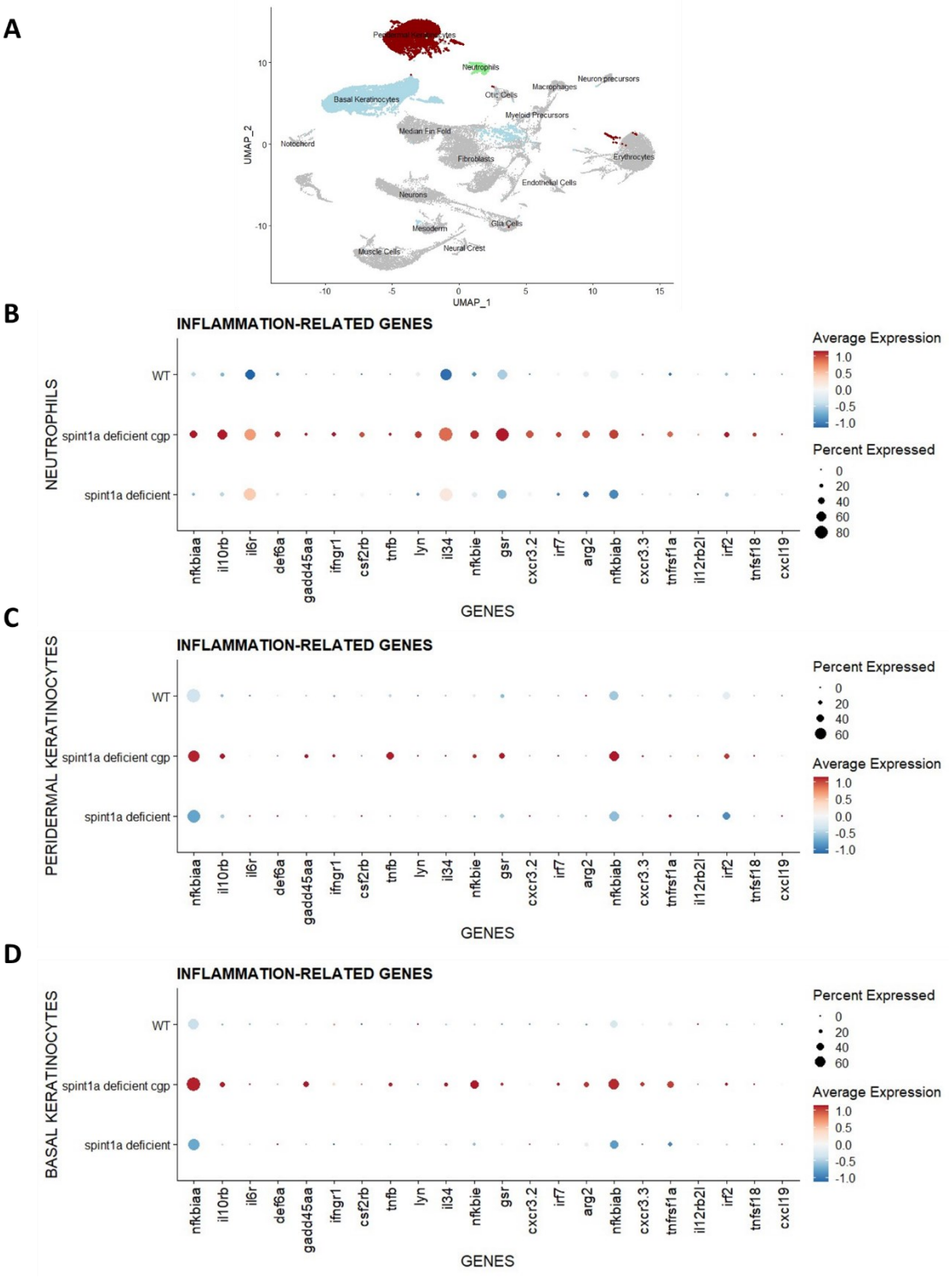

Figure 67. Expression profiles of inflammation-related genes induced by CGP3466B in different cell types. (A) UMAP representing the different cell clusters in the scRNA-seq, highlighting neutrophils, peridermal and basal keratinocytes in green, dark red and light blue, respectively. **(B)** Dotplot showing inflammation-related genes upregulated in neutrophils in response to the treatment with Gapdh nuclear translocation inhibitor CGP3466B. **(C)** Dotplot of peridermal keratinocyte expression of the same 22 genes related to inflammation that were overexpressed in neutrophils. **(D)** Dotplot representing basal keratinocyte expression of the same 22 genes upregulated in neutrophils after the treatment with CGP3466B. UMAP, Uniform Manifold Approximation and Projection.

As NF-κB did not regulate neutrophil infiltration into the inflamed skin and most inflammation-related genes were similarly induced in neutrophils and keratinocytes by inhibition of Gapdh nuclear translocation, the neutrophil subclusters were further analyzed with the aim of finding genes regulated in different neutrophil populations by Gapdh nuclear translocation inhibition (**Figure 68A**). We focused our attention in clusters 1 and 3, which mainly represent untreated and treated neutrophils of Spint1a-deficient larvae and, therefore, we could identify genes overexpressed in neutrophils of cluster 1 that decreased in cluster 3; that is, upon Gapdh nuclear translocation inhibition. With these criteria, two different genes were found: *il4* and protein tyrosine phosphatase non-receptor type 6 (*ptpn6*) (**Figure 68B**). The expression of these genes was drastically induced in neutrophils of *spint1a* -/- larvae and treatment with CGP3466B robustly attenuated it (**Figure 68C**). Notably, the expression of *il4* was restricted to neutrophil cluster 1, while *ptpn6* was also expressed in cluster 4, which was mainly integrated by neutrophils from untreated Spint1a-deficient larvae (**Figure 68D**). Furthermore, it was observed that *il4* expression was not expressed in other larval tissue, while *ptpn6* was mainly expressed in neutrophils, but also in macrophages (**Figure 68E**).

These results prompted us to study whether Il4 played a significant role in chronic skin inflammation using a Il4-deficient zebrafish line. The results showed that Il4 deficiency robustly alleviated the number of keratinocyte aggregates of Spint1a-deficient larvae (**Figure 69A-B**).

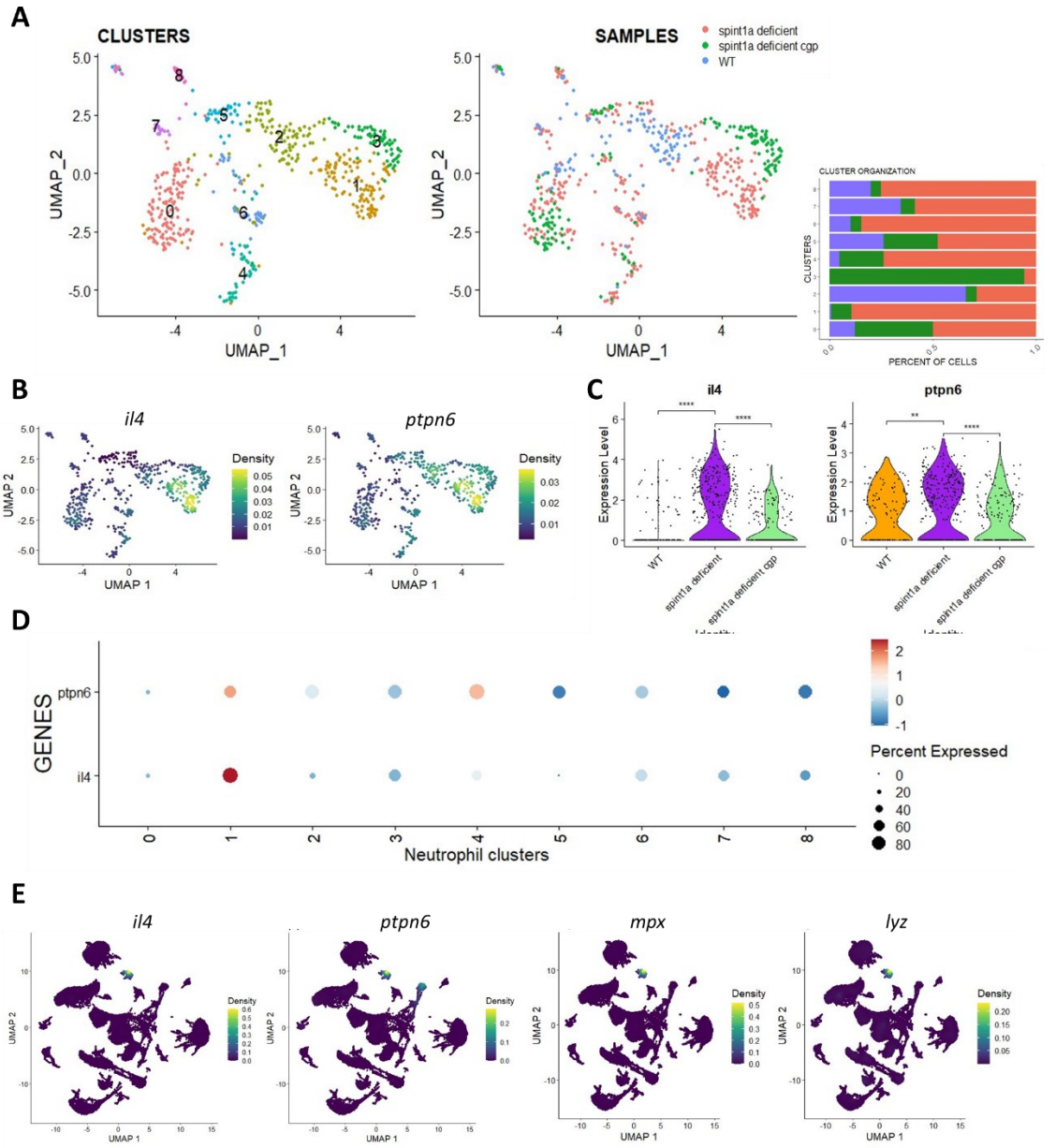

Figure 68. Neutrophil *il4* and *ptpn6* expression is regulated by Gapdh nuclear translocation. (A) UMAP representing the different neutrophil subclusters. Left, neutrophils are colored by cluster; right, neutrophils are colored by sample origin. The barplot shows the percentage of neutrophils of each sample forming each cluster. **(B)** Density plot showing the specific expression of *il4* and *ptpn6* in the neutrophils. **(C)** Violin plot showing expression of *il4* and *ptpn6* on each sample. **(D)** Dotplot representing the expression of *il4* and *ptpn6* in the different neutrophil clusters. **(E)** Density plot showing *il4, ptpn6, mpx and lyz* expression in all cell cluster of the scRNA-seq. Each point represents the gene expression level in a different cell analyzed. p-Values were calculated using Wilcoxon Rank Sum test. **p ≤ 0.01, ****p ≤ 0.0001. UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction; *il4,* interleukin-4*; ptpn6,* protein tyrosine phosphatase non-receptor type 6*; mpx,* myeloperoxidase*; lyz,* lysozyme.

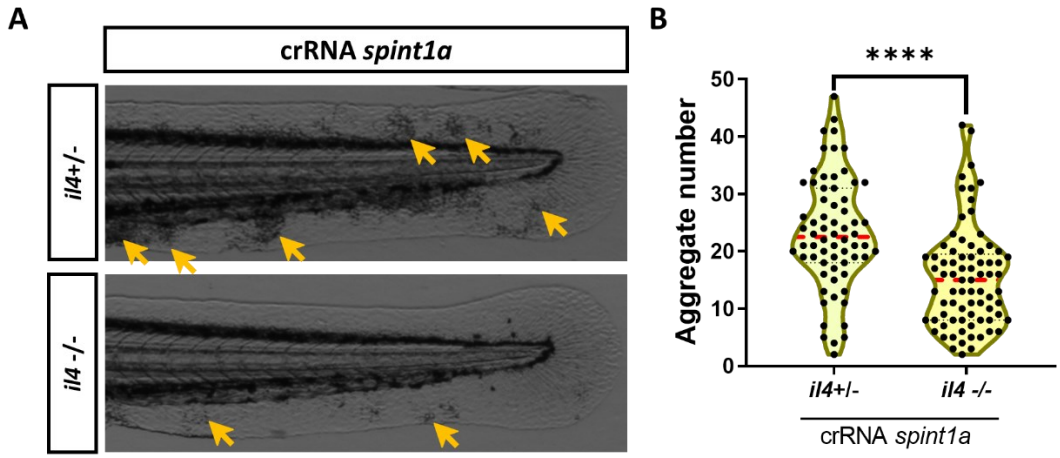

Figure 69. Neutrophil-derived Il4 is involved in the progression of chronic skin inflammation of Spint1a-deficient larvae. (A) Representative images showing the skin of *il4* +/- and *il4* -/- larvae upon genetic inhibition of Spint1a with CRISP-Cas9 technology. Yellow arrows label the keratinocyte aggregates in the skin. **(B)** Violin plot representing the number of keratinocyte aggregates in the skin of *il4* +/- and *il4* -/- larvae. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ****p ≤ 0.0001. crRNA, CRISPR RNA; *il4*, interleukin 4.

· Discussion

In this chapter, we have been able to determine that Gapdh nuclear translocation triggered *il4* expression in neutrophils of *spint1a* -/- larvae which, in turn, aggravated skin inflammation. Furthermore, pharmacological inhibition of Gapdh nuclear translocation regulated the expression of several inflammation-related genes in neutrophils and peridermal and basal keratinocytes.

Among these genes, *il4* and *dusp5* were found to be downregulated, while *nfkbiaa, pim2* and *plekhf1* were upregulated in Spint1a-deficient larvae treated with the Gapdh nuclear translocation inhibitor CGP3466B. Although we have focused our attention on Il4, it would be interesting to study the roles of these genes in the regulation of neutrophil behavior and the mechanisms by which nuclear Gapdh regulates them during skin inflammation. For example, *plekhf1* has been recently related to the autophagy process in neutrophils and T cells during the Kawasaki disease (Zhu, Xu, Hu, Li, & Liao, 2023) and autophagy regulation is one of the reported Gapdh non-canonical roles (Dhiman et al., 2023; Iqbal et al., 2021).

With the aim of clarifying the impact of Gapdh nuclear translocation on neutrophils, the neutrophil cluster was reclustered, obtaining a total of nine different clusters. These clusters share some features with the clusters identified in chapter 2. WT neutrophils were predominantly included in the cluster 2, characterized by the expression of general neutrophil markers, like *lyz* and *mpx*. Curiously, this cluster was also characterized by the high transcript levels of *gapdh*. Notably, cluster 1 was almost specific for neutrophils from untreated *spint1a* -/- , while cluster 3 was enriched in mutant neutrophils responding to the treatment with the inhibitor CGP3466B. In cluster 3, one of the most upregulated gene was *nfkbiaa*, which encodes the inhibitor of NF-κB Ikabaa (Jardin, 2022). In fact, treatment of *spint1a* -/- larvae with Gapdh nuclear translocation inhibitor resulted in reduced NF-κB activity in the skin. This is not unexpected, since GAPDH was reported to act as an enhancer of NF-κB activity in non-Hodgkin's B lymphoma and in human angioimmunoblastic T cell lymphoma (Chiche et al., 2015; Mondragon et al., 2019). Furthermore, inhibition of GAPDH/SIAH1 nuclear translocation with silvelestat sodium inhibits progression of Spinal Cord Injury (SCI) by decreasing the activation of NF-κB (Huo et al., 2016). Similarly, we found that inhibition of Gapdh nuclear translocation with CGP3466B increased the levels of *nfkbiaa*, leading to decreased NF-κB activity, indicating that Gapdh nuclear translocation triggered NF-κB activity. Additionally, several genes related to inflammation were induced after the treatment with CGP3466B in neutrophils, such as *nfkbie*, which encodes Ikbe, another inhibitor of NF-κB (Mansouri et al., 2016); Il-10 receptor B (*il10rb*) and *def6* , which have been reported as anti-inflammatory genes (Binder et al., 2017; Shouval et

al., 2014; Vistica et al., 2012); and Il6r, which may mediate both anti and pro-inflammatory roles (Muneer, 2016). These results suggest that Gapdh nuclear translocation plays a pro-inflammatory role in neutrophils of the *spint1a* -/- model through the regulation of NF-κB. Nevertheless, genetic inhibition of NF-κB in neutrophils failed to alleviate skin inflammation of e Spint1a-deficient larvae. These results suggest, therefore, that although Gapdh nuclear translocation activate NF-κB in neutrophils, this did not contribute to the progression of chronic skin inflammation. However, as inhibition of Gapdh nuclear translocation showed a similar effect on peridermal and basal keratinocytes and reduced skin NF-κB activity, pharmacological modulation of Gapdh nuclear translocation could be a potential treatment in inflammatory diseases where NF-κB has been found to be directly involved on their progression, such as atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, psoriasis and atopic dermatitis, among others (Mellett, 2020; M. H. Park & Hong, 2016).

The scRNA-seq analysis pointed out *il4* and *ptpn6* as potential genes regulated by Gapdh nuclear translocation only in neutrophils, that could be altering their behavior. Ptpn6, also known as Src homology 2 domain-containing protein tyrosine phosphatase 1 (Shp1), is a protein which is physically associated with the IL-4 receptor alpha-chain (IL-4Rα) and has been shown to play both positive and negative regulation of IL-4 signaling (H. Huang & Paul, 2000; Z. Huang et al., 2005). It is tempting to speculate, therefore, that Ptpn6 positively Il4 signaling in zebrafish neutrophils. IL-4 is an antiinflammatory cytokine mainly produced by basophils that has been shown to regulate B cells, ILCs, macrophage polarization to M2 and T cell polarization to T_H2 (Yamanishi & Karasuyama, 2016). IL-4 has been reported as a crucial factor in the establishment of the inflammation resolution by eosinophils in a toll-like receptor 2-mediated paw inflammation mouse model (Kolbinger et al., 2023). However, to the best of our knowledge, the production of IL-4 by mammalian neutrophils has never been reported. We found that in larvae with inflamed skin, *il4* transcript levels were dramatically induced in neutrophils and pharmacological inhibition of Gapdh nuclear translocation robustly attenuated them. This *il4* expression in neutrophils was also reported in a recent study (Garcia-Lopez et al., 2023). Moreover, Il4 deficiency strongly reduced the number of keratinocyte aggregates. This not surprising, since the IL-4Rα has been broadly studied in atopic dermatitis disease, where T cells and keratinocytes were both found to express it and to respond to IL-4 (Bangert et al., 2021; Junghans, Jung, & Neumann, 1996; Timms, Guo, Arkwright, & Pennock, 2022). In addition, IL-4 induces the expression of IL-4Rα in keratinocyte, resulting in a positive feedback loop that amplifies inflammation (Junghans et al., 1996). In agreement with these results, we also found that epidermal keratinocytes expressed *il4r.1*, suggesting that the Il4 produced by neutrophils

might directly regulate keratinocyte hyperproliferation and skin inflammation. Further research should be done to test this hypothesis. e interaction between the Il-4 released by the neutrophils and the Il-4 receptor expressed in keratinocytes. Collectively, all these results point out to neutrophil-specific overexpression of *il4* as a key mediator of the progression of chronic skin inflammation in the Spint1a-deficient zebrafish model.

It would also been interesting to reveal the molecular mechanism involved in the regulation of il4, and other inflammation-related genes, by Gapdh. One possibility could be that Gapdh regulates these inflammatory genes by binding to the 3'UTR of their mRNAs (Garcin, 2019; Nagy & Rigby, 1995). Thus, the inhibitor of Gapdh nuclear translocation promotes its cytosolic accumulation where it would bind the 3'UTR of these inflammatory genes, stabilizing and increasing their mRNA levels. Alternatively, Gapdh might directly regulate gene expression by either interacting and activating P300/CBP complex or with OCT1 and the androgen receptor (Harada et al., 2007; Nicholls, Li, et al., 2012).

To summarize, this chapter has allowed us to better understand how Gapdh nuclear translocation regulates neutrophil behavior in the *spint1a* -/- model (**Figure 70**). Until now, all the results obtained with Gapdh has been in a chronic skin inflammatory context, so it would also be interesting to study whether this mechanism is exclusive of chronic skin inflammation or also operate in homeostasis.

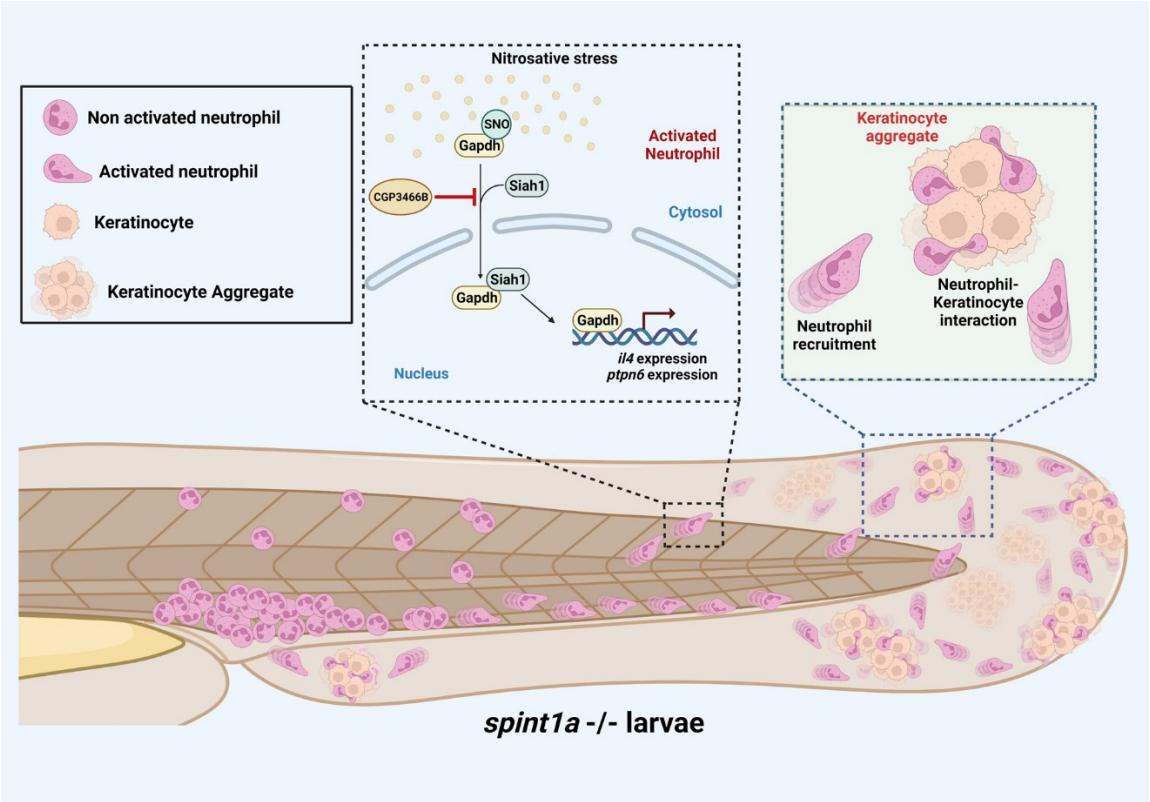

Figure 70. Gapdh nuclear translocation regulates neutrophil behavior in Spint1a-deficient larvae. Gapdh nuclear translocation triggers the expression of genes like il4 and ptpn6, activating neutrophil recruitment to the keratinocyte aggregates in the skin, increasing speed and displacement length. Once in the aggregates, neutrophils interact with the keratinocytes during long periods of time. Inhibition of Gapdh nuclear translocation decreases the number of neutrophils recruited to the skin, the speed and displacement length of these neutrophils, and the time they are interacting with the keratinocytes.

Chapter 5: Gapdh nuclear translocation regulates neutrophil migration in response to an acute inflammation.

· Results

The main objective of this chapter was to determine whether neutrophil recruitment is controlled by Gapdh nuclear translocation not only in chronic but also in acute inflammation. For this reason, we tested the effects of CGP3466B in a zebrafish wounding model extensively used. For that, in a first approach, 3 dpf zebrafish larvae were treated during 3h with the inhibitor CGP3466B at a concentration of 10 µM, and then they were wounded, cutting with a razor blade the final part of their tail. In this model, the peak of neutrophils recruitment after a tail wound is reached between 4 and 6 h post-wounding (hpw) (de Oliveira et al., 2013), so we performed our analysis at 5 hpw (**Figure 71A**). The results showed that the number of neutrophils recruited to the wounded tail was considerably reduced at 5 hpw when the larvae were previously treated with the Gapdh nuclear translocation inhibitor CGP3466B (**Figure 71B-C**). In addition, CGP3466B-treated larvae showed reduced number of total neutrophils. For this reason, instead of measuring the total number of neutrophils in the wound, it was decided to quantify the percentage of recruited neutrophils. It was also found that the treatment with CGP3466B resulted in reduced neutrophil recruitment at 5 hpw (**Figure 71D**).

We next performed a dynamic study of the neutrophil migration to wound by recording the larvae for 5 h (**Supplementary videos 6 and 7, Figure 72A**). At 0 hpw no difference in the percentage of neutrophils recruited to the wound was observed. The difference started to be observed as soon as 1 hpw, when the DMSO-treated larvae showed a slightly increased percentage of neutrophils recruited. The differences increased over the time, observing the most significant difference at 5 hpw, coinciding with the peak of neutrophil recruitment in the wound (**Figure 72B-C**). Moreover, it was also observed that while the percentage of recruited neutrophils increased from 4 to 5 hpw in DMSO-treated larvae, it no longer increased in the CGP3466B-treated group (**Figure 72C**).

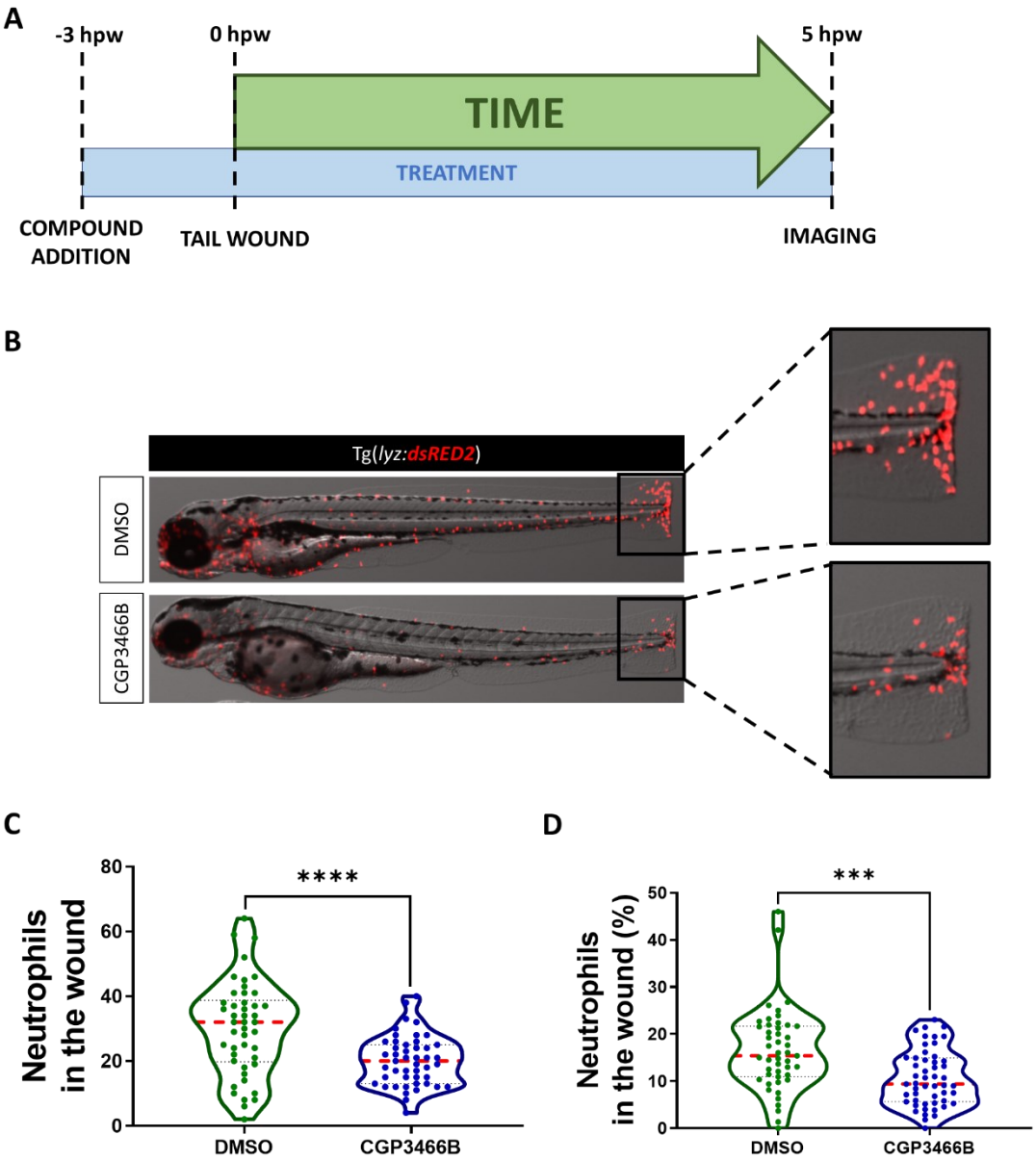

Figure 71.Pharmacological inhibition of Gapdh nuclear translocation impairs neutrophil recruitment to wound. (A) Three dpf larvae pre-treated for 3 h with 10 µM CGP3466B were wounded in the tail and imaged and the neutrophils recruited were quantified at 5 hpw. **(B)** Representative merged images of the larvae and the neutrophils of DMSO- (control) and CGP3466B-treated larvae at 5 hpw. **(C)** Total number of neutrophils recruited to the wound. **(D)** Percentage of recruited neutrophils at 5 hpw. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. ***p ≤ 0.001, ****p ≤ 0.0001. Hpw, h post-wounding; DMSO, Dimethyl sulfoxide.

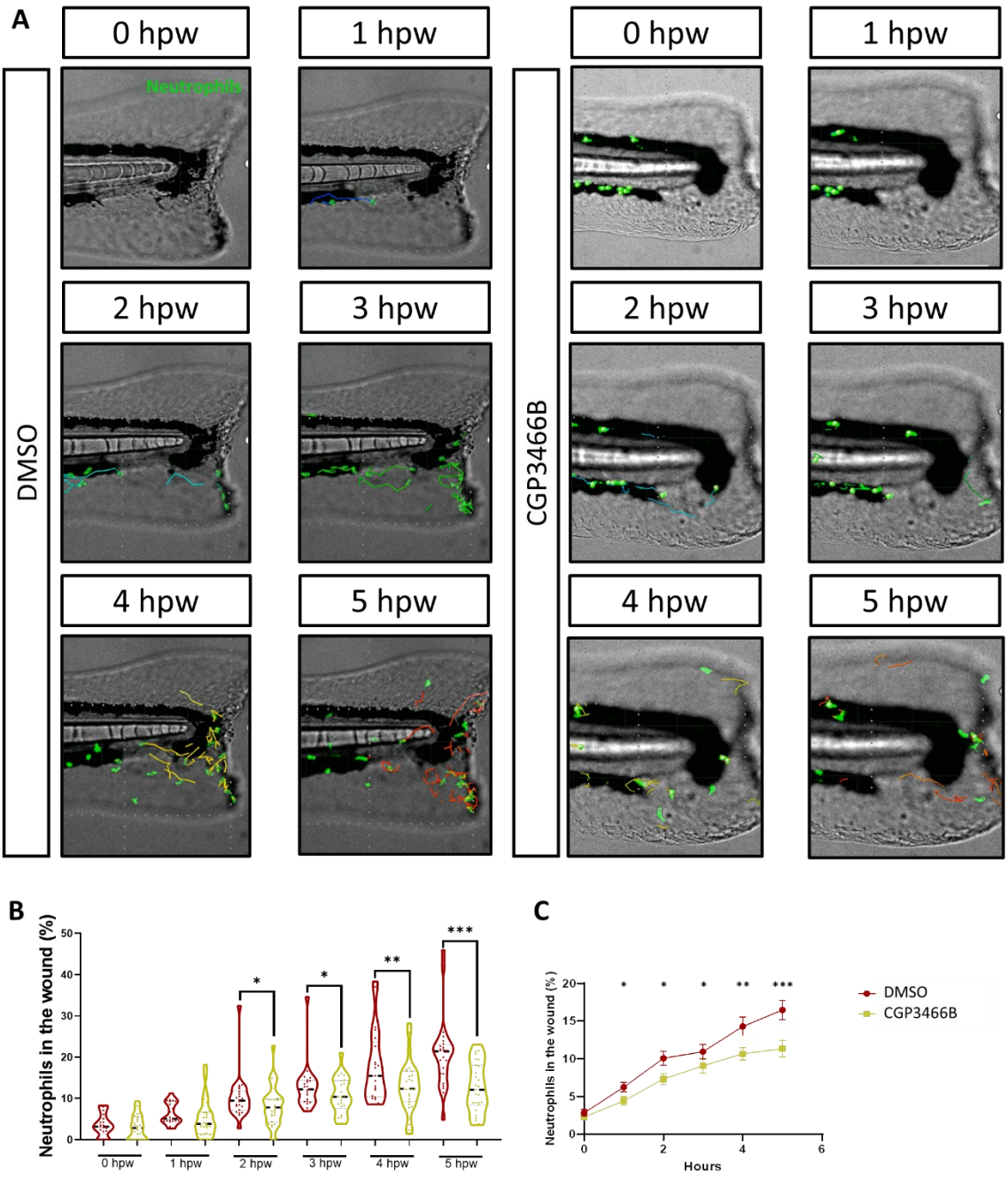

Figure 72. Neutrophil recruitment dynamics are altered after the treatment with CGP3466B. (A) Representative merged images showing the tails, the neutrophils and their tracks in dragon tail format of DMSO-treated and CGP3466B-treated larvae at 0, 1, 2, 3, 4 and 5 hpw. **(B, C)** The percentage of neutrophils recruited to the tail was measured at each time point and condition. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test comparing the two samples at the same time point. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Hpw, hours post wound; DMSO, dimethyl sulfoxide.

The movies recorded by long-term imaging of the neutrophil recruitment to the wound in DMSO-treated and CGP3466B-treated larvae were processed in order to analyze other different parameters related to the neutrophil migration. Neutrophil movement was tracked during the 5h of movie, extracting parameters like the mean speed of their movement and the mean length

of the displacement to migrate to the wound in each condition. By analyzing the tracks of the different neutrophils (**Figure 73A**), it was observed that neutrophils moved slower (**Figure 73B**) and that the displacement length also decreased after the treatment with CGP3466B (**Figure 73C**).

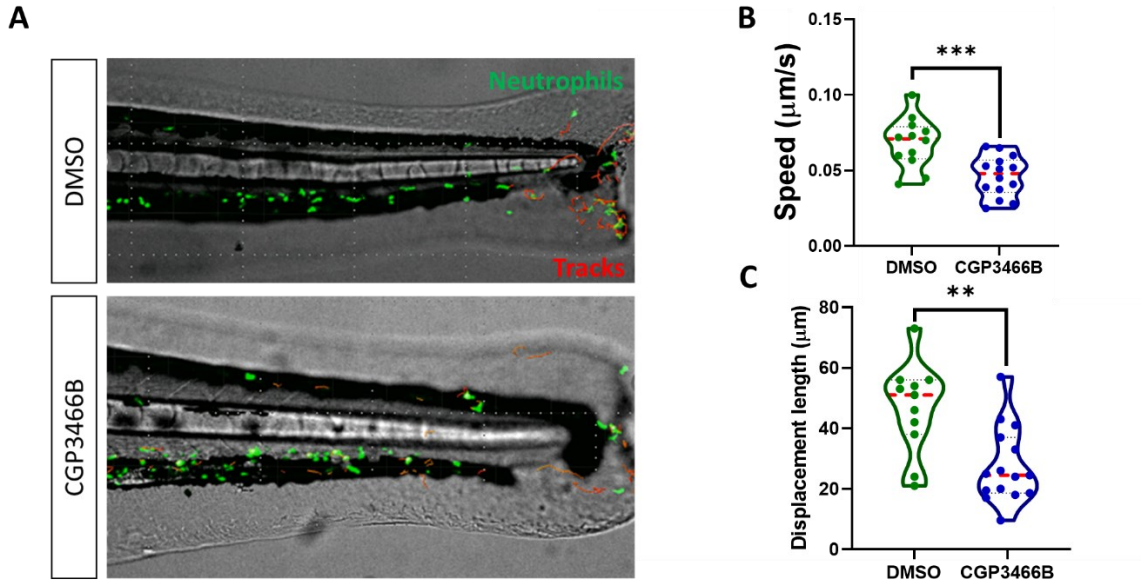

Figure 73. Pharmacological inhibition of Gapdh nuclear translocation alters neutrophil recruitment to wound. (A) Representative merged images showing the tails, neutrophils, and their respective tracks in dragon tail format of DMSO-treated and CGP3466B-treated larvae at 5 hpw are shown. **(B)** The mean speed of the movement of all the neutrophils was measured, showing a decrease in the larvae treated with CGP3466B 10 µM. **(C)** The length of the displacement of all the neutrophil was measured by the analysis of their tracks, showing also a decrease carried out by the treatment with CGP3466B. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. **p ≤ 0.01, ***p ≤ 0.001. Hpw, hours post wound; DMSO, dimethyl sulfoxide.

The signals that control early neutrophil recruitment are completely different to those which trigger the amplification of neutrophil recruitment, in some conditions known as “swarming” (de Oliveira et al., 2016; Poplimont et al., 2020). For this reason, the neutrophil migration to the wounds was separated into two different time windows. The period of time between 0 and 2 hpw was considered the “early recruitment” phase, when signals such as H_2O_2 drive the neutrophil mobilization and recruitment to the injury (de Oliveira et al., 2016). The period of time between 3 and 5 hpw was considered a “late recruitment” phase, where other signals, such as Cxcl8 family chemokines, Cxcl1 and LTB4, released by other immune cells, drive the amplification of the recruitment of neutrophils (de Oliveira et al., 2016). Taking this into consideration, neutrophil migration movies were cropped and the early and late neutrophil recruitment was analyzed individually, in order to know whether Gapdh nuclear translocation affected the signals involved in early and/or late neutrophil recruitment (**Figure 74A**). During the early recruitment, the neutrophils of the larvae treated with CGP3466B showed decreased speed

and displacement length (**Figure 74B**). Interestingly, it can be observed that neutrophils of CGP3466B-treated larvae showed delayed recruitment and took more time to respond to wound (Supplementary videos 6 and 7). In addition, the differences in the speed of both groups strongly increased during late recruitment, i.e. between 3 and 5 hpw (**Figure 74B**). However, the difference in the displacement length of the neutrophil tracks of both groups was similar during early and late recruitment (**Figure 75C**).

We next wonder whether Gapdh nuclear translocation was regulating neutrophil random migration rather than chemotaxis to a specific stimulus. For that, 3 dpf larvae were pre.treated for 3h with CGP3466B and neutrophils of their heads were recorded for 5 h (**Figure 75A and Supplementary videos 8 and 9**). In this case, neither the speed nor the displacement length was altered after the treatment with CGP3466B, indicating that Gapdh nuclear translocation does not regulate neutrophil random migration (**Figure 75B-C**).

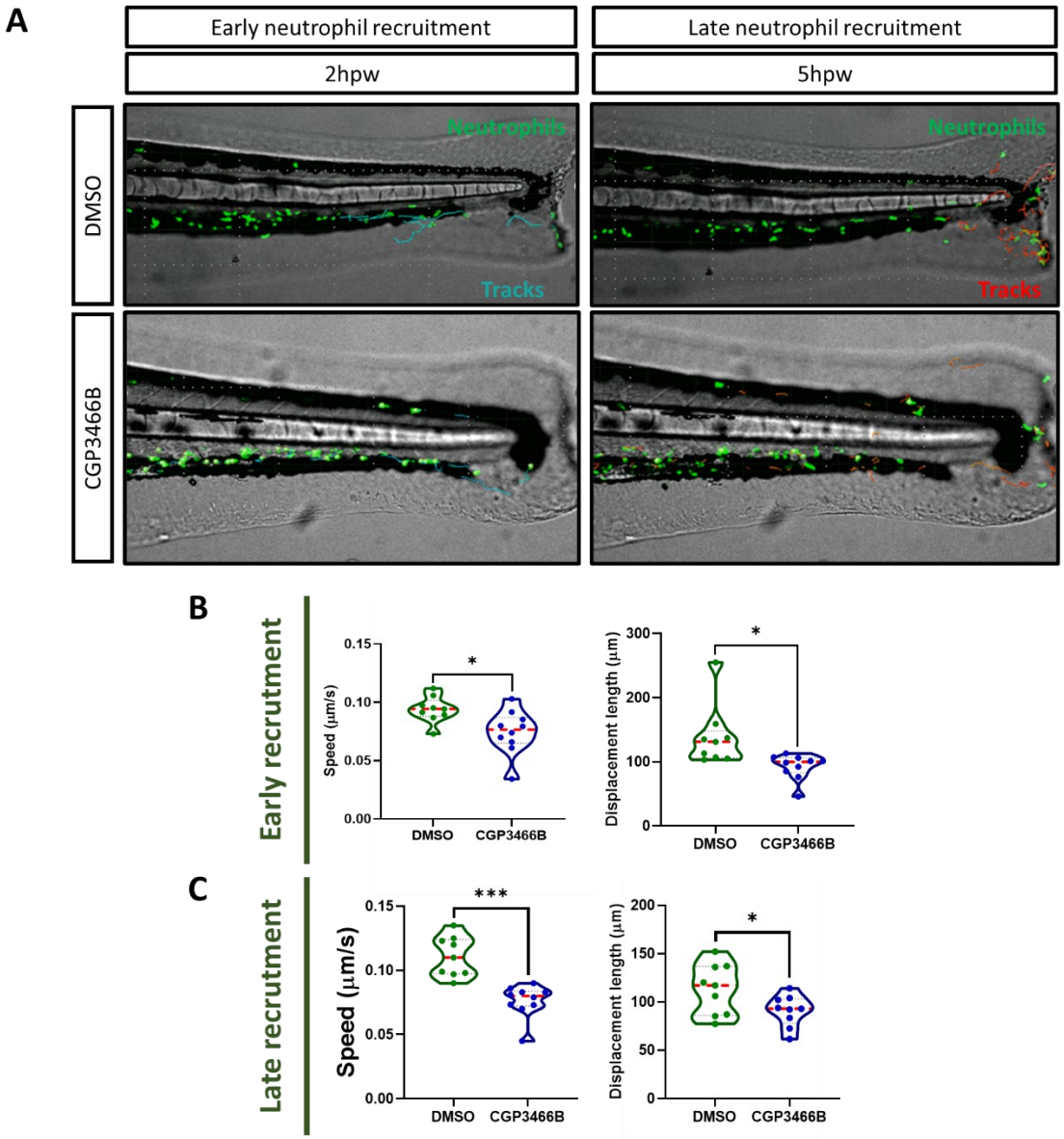

Figure 74. Impact of pharmacological inhibition of Gapdh nuclear translocation in the early and late neutrophil recruitment phases to tail wounding. (A) Representative merged images shown the tails, the neutrophils and their tracks in dragon tail format of DMSO and CGP3466B-treated larvae at 2 hpw (end of the early recruitment phase) and at 5 hpw (end of late recruitment phase). **(B, C)** Speed and displacement length during early (B) and late (C) neutrophil recruitment phases. Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test comparing the two samples at the same time point. **p ≤ 0.01, ***p ≤ 0.001. Hpw, h post-wounding; DMSO, dimethyl sulfoxide.

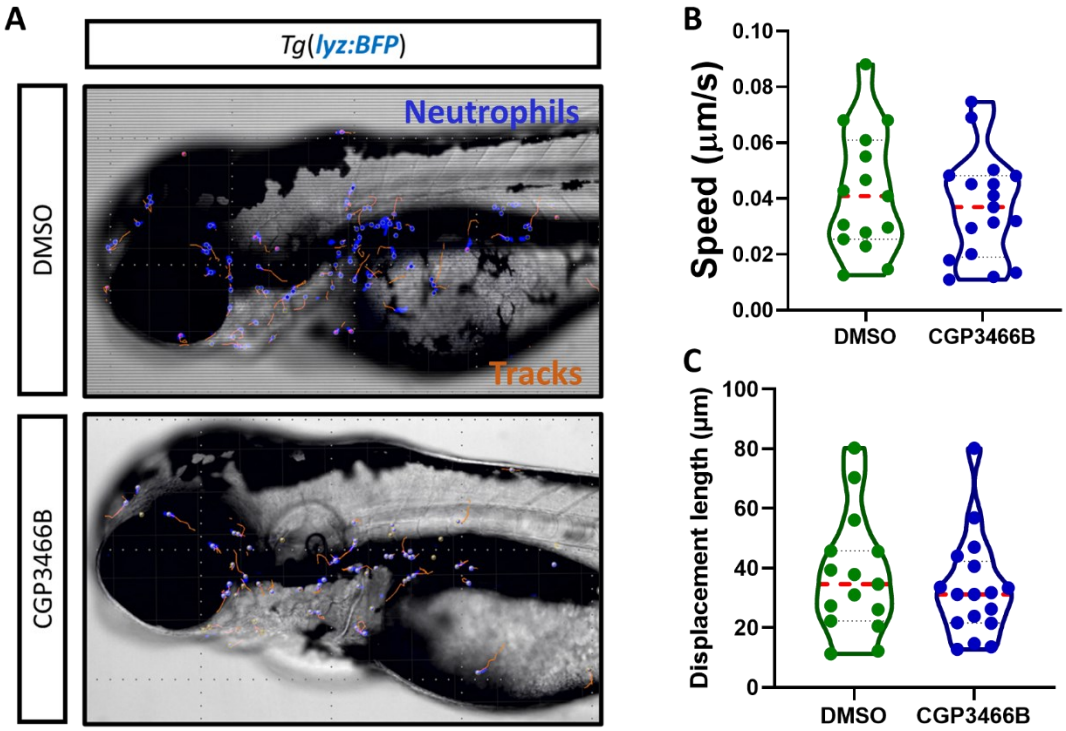

Figure 75. Random neutrophil migration is not regulated by Gapdh nuclear translocation. (A) Representative images of the heads, the neutrophils and their tracks in dragon tail format of DMSO-treated (control) and CGP3466B 10 µM-treated 3dpf larvae. **(B, C)** Speed (B) and displacement length (C). Each dot represents one individual, and the median for each group is also shown. p-Values were calculated using t test. **p ≤ 0.01, ***p ≤ 0.001. Hpw, hours post wound; DMSO, dimethyl sulfoxide.

· Discussion

In this chapter, Gapdh nuclear translocation has been shown to be involved in the regulation of neutrophil migration in acute inflammation. As the inhibition of its nuclear translocation with CGP3466B decreased neutrophil migration in response of an acute inflammatory stimulus, it is tempting to speculate that nuclear Gapdh regulates neutrophil migration to wound. Therefore, Gapdh nuclear translocation plays a crucial role in the regulation of neutrophil recruitment in both chronic and acute inflammation. Importantly, Gapdh did not alter neutrophil random migration, suggesting that Gapdh nuclear translocation specifically regulates neutrophil migration in the context of inflammation. This is not surprising, since Gapdh is translocated to the nucleus by inflammation and nitrosative stress upon its interaction with SIAH1 (Kornberg et al., 2010; Muronetz et al., 2021; Sirover, 2021) and Spint1a-deficient larvae showed high levels of nitrosative stress and genetic inhibition of Siah1 ameliorated skin inflammation in this model (see Chapter 1).

Another interesting observation is that although Gapdh nuclear translocation was involved in both early and late neutrophil recruitment, it had a higher impact in late neutrophil recruitment. There could be explain in different ways. Firstly, Gapdh regulates neutrophil migration in a cell autonomous manner and, therefore, no matter the recruitment phase. Alternatively, Gapdh regulates the release of chemoattractant molecules release in early and/or late recruitment phases, such as H_2O_2, Cxcl8 or LTB4. H_2O_2 is an strong candidate, since it rapidly released, as soon as 3 min, in the injured skin reaching a peak at 20 min (Niethammer, Grabher, Look, & Mitchison, 2009), promotes early neutrophil recruitment through the SRC kinase LYN (de Oliveira et al., 2016) and has can oxidates GAPDH in its residue Cys^{149}, situated in the catalytic site, inhibiting its glycolytic function and promoting its non-canonical roles (Arutyunova et al., 2003; Talwar et al., 2023). Future scRNA seq and cell specific Gapdh genetic inhibition experiments will clarify this issue.

Another important signal for neutrophil recruitment is Cxcl8 and its interaction with its receptors has been broadly confirmed in zebrafish to regulate neutrophil late recruitment (de Oliveira et al., 2016; Sarris et al., 2012). In atopic dermatitis, the expression of *CXCL8* and GAPDH positively correlates (T. Wang, Zhang, Li, Qi, & Zhang, 2021) and, moreover, a peptide derived from GAPDH to epithelial cells in response to fungal infection increases the release of CXCL8 and is able to clear the infection (Wagener et al., 2013). Experiments like CUT&RUN or ChIP could help elucidating if GAPDH nuclear translocation is controlling the expression of Cxcl8 and other inflammatory genes. Alternatively, Gapdh may also regulate the stability and translation efficiency of the mRNA of inflammatory cytokines (Garcin, 2019).

Leukotrienes are another family of signals involved in the amplification of neutrophil recruitment. LTB4 is the most broadly studied in the neutrophils field. Gradients of LTB4 trigger the recruitment of new neutrophils to the inflamed tissues (Afonso et al., 2012), and also helps neutrophils during the colonization of distant tissues in some diseases like cancer (Wculek & Malanchi, 2015). Apart from LTB4, experiments using leukotriene receptor antagonists showed that other leukotrienes like LTC4, LTD4 and LTE4 are also involved in neutrophil recruitment (Yano, White, Sampson, Wormley, & Fidel, 2021). GAPDH was reported to bind LTC4 in its Rossmann Fold domain, altering its function, and due to the similar structure, the other leukotrienes are also predicted to bind GAPDH (Puder & Soberman, 1997). Further research should be done to clarify if the binding of GAPDH to leukotrienes may also affect skin inflammation.

In summary, in we found that Gapdh nuclear translocation is an important factor during neutrophil recruitment in acute and chronic skin inflammation (**Figure 76**). However, the mechanism involved need to be determine in future studies.

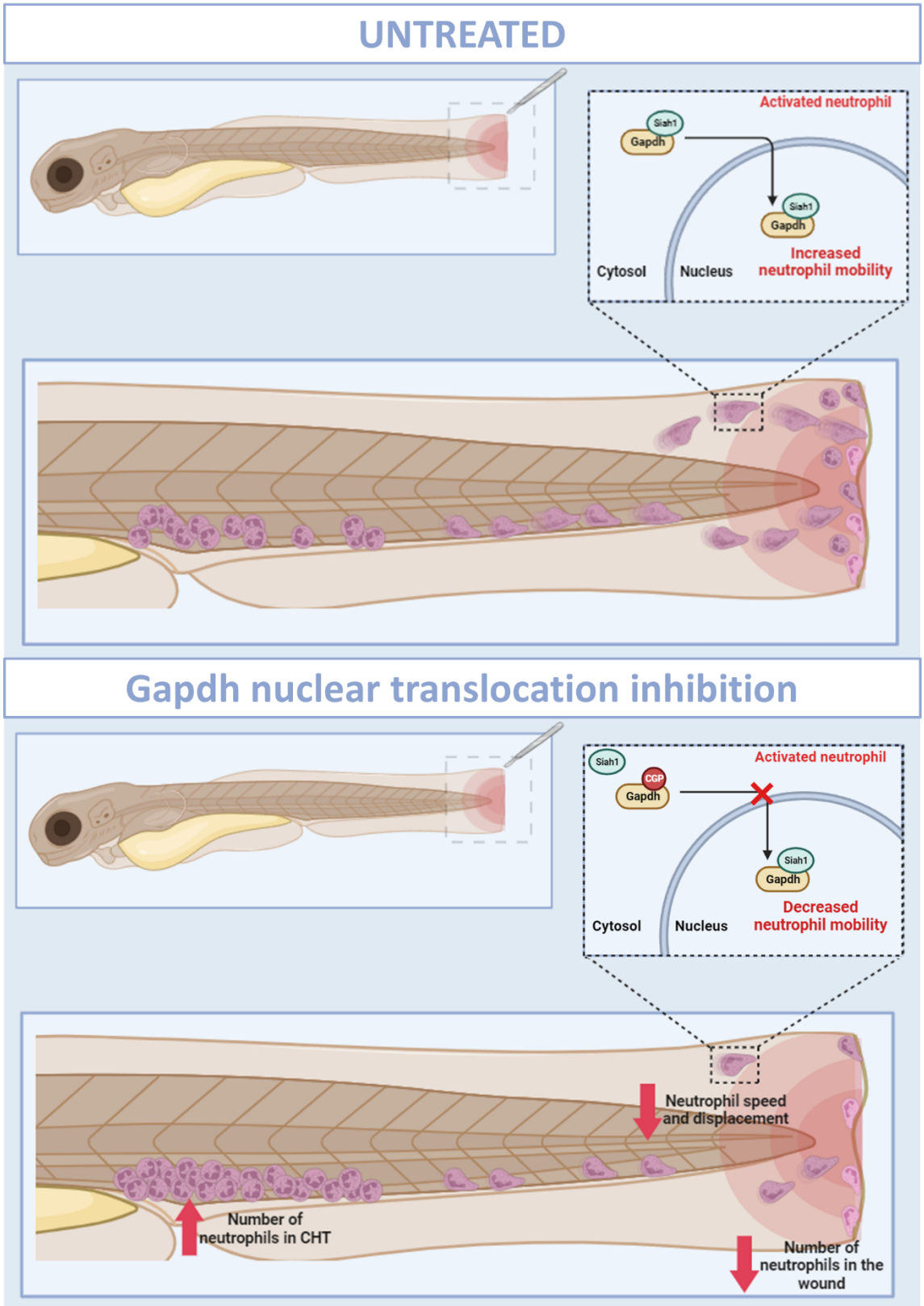

Figure 76. Gapdh nuclear translocation regulates neutrophil recruitment in acute inflammation. Gapdh nuclear translocation inhibition impacts neutrophil recruitment to the wound, reducing the number of neutrophils recruited, the speed and displacement of the neutrophils. This situation increases the number of neutrophils that keep retained in the CHT. CHT, Caudal Hematopoietic Tissue.

CONCLUSIONS

1. Spint1a-deficient zebrafish larvae display increased oxidative and nitrosative stress in the skin and both promote chronic skin inflammation characterized by neutrophil infiltration and keratinocyte aggregates.
2. Gapdh is overproduced in the skin of Spint1a-deficient zebrafish larvae and in skin lesions of human psoriasis patients. Its nuclear translocation is associated with keratinocyte cell death and hyperproliferation. Pharmacological inhibition of GAPDH nuclear translocation reduces skin inflammation in both Spint1a-deficient zebrafish larvae and human organotypic 3D skin models of psoriasis.
3. The mRNA of *gapdh* is specifically expressed in neutrophils and muscle cells. Genetic and pharmacological inhibition of Gapdh alleviates skin inflammation of the Spint1a-deficient zebrafish model. However, *gapdhs* is ubiquitously expressed and its inhibition does not affect skin inflammation.
4. There is a positive correlation between the number of skin infiltrated neutrophils and the number of keratinocyte aggregates in the Spint1a-deficient zebrafish model. In addition, neutrophils exacerbate chronic skin inflammation in this model.
5. Pharmacological and genetic inhibition of Gapdh nuclear translocation reduces neutrophil recruitment, speed and the interaction time with keratinocyte aggregates in the Spint1a-deficient zebrafish model. In addition, genetic experiments reveal a cell-autonomous effect of Gapdh on neutrophil biology.
6. Gapdh nuclear translocation regulates the expression of various genes, particularly those associated with inflammation, immune response, and gene regulation, in the Spint1a-deficient zebrafish model.
7. Pharmacological inhibition of Gapdh nuclear translocation reduces NF-κB activity in the skin of Spint1a-deficient zebrafish larvae and alleviates skin inflammation.
8. Gapdh nuclear translocation induces *il4* expression in neutrophils in Spint1a-deficient zebrafish larvae and neutrophil-derived Il4 exacerbates chronic skin inflammation in this model.
9. Pharmacological inhibition of Gapdh nuclear translocation reduces neutrophil recruitment, speed and displacement length to the wounded tail of zebrafish larvae.

RESUMEN

Las enfermedades inflamatorias crónicas representan una de las mayores causas de muerte en el mundo, siendo responsables de tres de cada cinco fallecimientos en el mundo (Pahwa et al., 2023). Estas patologías se pueden generar cuando el sistema inmune no es capaz de eliminar adecuadamente el microorganismo infeccioso o material externo que está desencadenando una inflamación aguda, causando vasodilatación, aumento de la permeabilidad vascular y reclutamiento de leucocitos (M. Li et al., 2021). Cuando este proceso se mantiene de forma persistente, puede originar un tejido fibrótico y fibrosis, que genera disfunción y fallos orgánicos (Henderson et al., 2020).

Este tipo de enfermedades pueden originarse en los distintos órganos y tejidos del organismo, como en la piel, una de las principales barreras del organismo ante agentes exógenos organizada en tres capas: epidermis, dermis e hipodermis (Kabashima et al., 2019). Entre las enfermedades inflamatorias crónicas que afectan a la piel encontramos dos principales: psoriasis y dermatitis atópica. La psoriasis es una enfermedad inmunomediada que afecta a un porcentaje de la población del 2-3% y se caracteriza por una desregulación de la hiperproliferación de los queratinocitos de la piel, seguida de la iniciación del sistema inmunitario, implicado en la secreción de citoquinas inflamatorias que, si perduran en el tiempo, pueden llegar a afectar a otros órganos y tejidos del organismo a parte de la piel (Greb et al., 2016; Lebwohl, 2003). Por su parte, la dermatitis atópica, también conocida como eczema, es una enfermedad inflamatoria crónica que presenta diversas manifestaciones, pero generalmente da lugar a piel seca y liquenificación en el 15-20% de los niños y un 10% de los adultos en el mundo (Kolb & Ferrer-Bruker, 2023; Stander, 2021). En ambas enfermedades, los leucocitos toman un papel crucial, encargándose de reclutar al resto de células del sistema inmunitarias, como los linfocitos T, y controlar su respuesta, T_H17 y T_H22 en el caso de la psoriasis, y T_H2 en el caso de la dermatitis atópica (Greb et al., 2016; Leung & Bieber, 2003).

Los neutrófilos son los leucocitos más abundantes en la sangre, representando un 70% de los leucocitos circulantes. Estas células son las primeras en ser reclutadas a un estímulo inflamatorio, representando uno de los principales mecanismos de defensa del organismo (Kolaczkowska & Kubes, 2013; Phillipson & Kubes, 2019). Su acumulación a largo plazo en los tejidos u órganos inflamados puede generar daños irreparables en los mismos (Brewer, 2023). Tanto en psoriasis como en dermatitis atópica, los neutrófilos presentan niveles por encima de la media (Carmona-Rivera & Kaplan, 2013; Dogru & Citli, 2017). Aunque su rol en dermatitis atópica ha sido, hasta

ahora, poco estudiado y principalmente relacionado con el desarrollo de picor en la piel (Walsh et al., 2019), en psoriasis sí se ha estudiado mucho más su importancia. Hasta ahora, se ha descrito que los neutrófilos son reclutados gracias a citoquinas como CXCL8, CXCL10 o CCL5 secretadas por los queratinocitos activados. Estos neutrófilos reclutados, una vez en la zona inflamada caracterizada por la hiperproliferación de queratinocitos, son capaces de secretar IL17A (R. Keijsers et al., 2014; R. R. Keijsers et al., 2014), responsable de la posterior respuesta T_H17 de los linfocitos T, expresan genes relacionados con estrés oxidativo, como *MPO* o *NOX2*, y también son capaces de llevar a cabo el proceso de NETosis contra los queratinocitos activados (Chiang et al., 2019).

La enzima Gliceraldehído 3-Fosfato Deshidrogenasa (GAPDH) es un homotetrámero de 144 kDa formado por cuatro subunidades de 36 kDa cada una (Jenkins & Tanner, 2006). Su función principal descrita es participar en la ruta glucolítica, catalizando la oxidación y fosforilación de gliceraldehído 3-fosfato en 1,3-bifosfoglicerato, reacción reversible (Bruns & Gerald, 1976), siendo una enzima crucial para la producción de ATP y piruvato en las células (Nicholls, Li, et al., 2012). Tiempo después a su descubrimiento, se observó que la enzima era una diana de una gran variedad de modificaciones postraduccionales, entre las que encontramos nitrosilación, acetilación, fosforilación, metilación, N-acetil glucosilación, oxidación, succinación o S-sulfenilación (Cho et al., 2018; Kornberg et al., 2018; Kornberg et al., 2010; Sirover, 2014, 2021; Talwar et al., 2023). Estas modificaciones post traduccionales generan cambios conformacionales de la enzima, alterando su función y permitiéndole llevar a cabo un gran abanico de funciones no canónicas (Garcin, 2019). De entre sus funciones no canónicas, muchas de ellas se han relacionado con inflamación. El gen *GAPDH* se ha visto sobreexpresado en una gran variedad de enfermedades inflamatorias (Chiche et al., 2015). La enzima se ha visto relacionada con procesos como el desencadenamiento de procesos de muerte celular como apoptosis, autofagia, transporte de hierro, actuando como factor de transcripción o modulador epigenético, regulando la estabilidad de mRNAs, o incluso actuando como un transportador de membrana (Chakravarti et al., 2010; Das et al., 2021; H. J. Yoo et al., 2022; J. Y. Zhang et al., 2015).

En neutrófilos, GAPDH se ha visto relacionada con el incremento en los niveles de proteínas como MAPK8 o MMP9 (Capuozzo et al., 2017), implicadas en migración; con la regulación de HIF-1α en condiciones de hipoxia, con el proceso de muerte celular, y más recientemente, con el proceso de NETosis junto a proteínas como PAD4 o elastasa (NE) (Awasthi et al., 2023).

Estudios previos de nuestro laboratorio han demostrado, usando el pez cebra como modelo de estudio de enfermedades y la línea *spint1a* -/- en particular para modelar inflamación crónica en

piel que, en enfermedades como la psoriasis o la dermatitis atópica, se da un exceso en el estrés oxidativo en los queratinocitos, produciendo un excesivo daño en el ADN de la célula. Este daño en el ADN desencadena un proceso de muerte celular dependiente de la enzima Poly ADP-Ribosa Polimerasa 1 (PARP1) llamado *parthanatos* (Arroyo et al., 2023; Martinez-Morcillo, Canton-Sandoval, Martinez-Menchon, et al., 2021; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021). Algunas publicaciones han relacionado una actividad no canónica de GAPDH: su translocación nuclear, con la hiperactivación de PARP1 y la muerte celular (Hara et al., 2005; Kim et al., 2006; Kornberg et al., 2010; N. Sen et al., 2008).

Para profundizar en el posible papel de GAPDH en inflamación crónica en piel, en este trabajo se han utilizado modelos de pez cebra, junto a cultivos 3D de queratinocitos y datos de pacientes. El pez cebra es actualmente un organismo modelo para el estudio de distintas enfermedades humanas debido a sus numerosas y conocidas ventajas.

Partiendo de esta base, se plantearon los distintos objetivos para este trabajo:

7. Estudiar el papel de GAPDH en inflamación crónica en piel, centrándonos en el impacto de su translocación nuclear, usando la línea deficiente en Spint1a de pez cebra y modelos organotípicos de piel en 3D como modelos de estudio de la psoriasis.
8. Analizar los patrones de expresión génica de varias poblaciones celulares a través de tecnología de secuenciación de ARN de célula única y, en particular, caracterizar los perfiles de expresión génica de queratinocitos peridermales y basales en larvas de pez cebra silvestres y deficientes en Spint1a.
9. Caracterizar el papel jugado por los neutrófilos en inflamación crónica en piel, usando el modelo de pez cebra deficiente en Spint1a.
10. Evaluar la influencia de la translocación de Gapdh al núcleo en el comportamiento de los neutrófilos en el contexto de inflamación crónica en piel usando como modelo la línea de pez cebra deficiente en Spint1a.
11. Analizar el perfil de expresión génica y las rutas de señalización reguladas por la translocación de Gapdh al núcleo en neutrófilos mediante el uso de secuenciación de ARN de célula única utilizando como modelo la línea de pez cebra deficiente en Spint1a.
12. Estudiar el impacto de la translocación de Gapdh al núcleo en el reclutamiento de neutrófilos en respuesta a estímulos inflamatorios agudos.

En primer lugar, hemos demostrado que en el modelo deficiente en Spint1a existe un exceso de estrés oxidativo y nitrosativo en la piel, en el que la familia de enzimas NADPH oxidasas (NOXs) es una de las principales responsables, gracias a la acción de enzimas como NOX1, NOX4 o NOX5.

Este fenómeno, junto con los datos anteriormente publicados con el exceso de muerte celular y la alteración del metabolismo de NAD+ en el modelo deficiente en Spint1a (Martinez-Morcillo, Canton-Sandoval, Martinez-Menchon, et al., 2021; Martinez-Morcillo, Canton-Sandoval, Martinez-Navarro, et al., 2021), nos llevó a la evaluación del impacto de enzimas que utilizan NAD^+ como cofactor en el desarrollo y transcurso de la inflamación crónica en piel. De entre ellas, GAPDH es una enzima comúnmente relacionada con metabolismo, muerte celular, y cuya actividad principal se ve modificada por el estrés oxidativo y nitrosativo (Colell et al., 2009; Garcin, 2019; J. Y. Zhang et al., 2015). En primer lugar, usando biopsias de pacientes de psoriasis, se observó una sobreproducción de GAPDH en peridermis y dermis no observada en los sujetos control, localizándose principalmente en la región nuclear de las distintas células observadas. Posteriormente, la inhibición de la translocación de Gapdh al núcleo en el modelo deficiente en Spint1a mostró una mejora en el fenotipo, reduciendo el número de agregados de queratinocitos, el número de neutrófilos dispersos, la actividad NF-κB, la muerte celular y la proliferación celular, características del modelo de estudio y de las enfermedades inflamatorias crónicas. La inhibición de la translocación de GAPDH al núcleo en modelos organotípicos de piel en 3D estimulados con IL22 e IL17A mostró una reducción en la expresión de genes inflamatorios típicos de psoriasis.

Seguidamente, estudios de secuenciación de ARN de célula única en el modelo deficiente en Spint1a nos permitieron observar que el ARNm de *gapdh* se expresa de forma específica en neutrófilos y células musculares, mientras que *gapdhs*, comúnmente conocida como una Gapdh2, se expresa de forma general en la mayoría de tipos celulares y su inhibición genética, al contrario de la de *gapdh*, no mostró cambios en la inflamación crónica en piel. Este resultado nos hizo centrarnos en los neutrófilos y su papel en enfermedades inflamatorias crónicas de la piel.

De este modo, observamos que cuanto mayor es el número de neutrófilos infiltrados en la piel, el número de agregados de queratinocitos se ve también incrementado en el modelo deficiente en Spint1a. Del mismo modo, la depleción de los neutrófilos en el modelo da lugar a una mejora del estado de la piel gracias a la disminución del número de agregados de queratinocitos. La inhibición de la translocación de Gapdh al núcleo mostró una alteración en el comportamiento de los neutrófilos, disminuyendo parámetros cinéticos como la distancia recorrida o la velocidad de movimiento, así como disminuyendo el número de neutrófilos que migran a los agregados de queratinocitos de la piel y el tiempo que neutrófilos y queratinocitos interaccionan. De este modo, se demuestra que la translocación de Gapdh está regulando la función negativa de los

neutrófilos en inflamación crónica en piel. Estos resultados fueron confirmados realizando la inhibición genética de *gapdh*.

Seguidamente, con el fin de comprender qué genes o rutas se están alterando con la inhibición de la translocación de Gapdh en neutrófilos en el modelo deficiente en Spint1a, estudios de secuenciación de ARN de célula única mostraron variaciones en la expresión de *il4*, gen inflamatorio que hasta ahora no se había relacionado con la actividad de los neutrófilos. La inhibición genética de *il4* en el modelo deficiente en Spint1a mostró una mejora significativa del estado de la piel, confirmando la implicación de la citoquina Il4, expresada en este contexto por los neutrófilos de forma específica.

Por último, hemos demostrado que la translocación de Gapdh al núcleo no solo está alterando el reclutamiento de neutrófilos en modelos de inflamación crónica. Experimentos de corte de cola simulando estímulos de inflamación aguda muestran que la inhibición de la translocación de Gapdh al núcleo disminuye considerablemente el número de neutrófilos que se reclutan a la herida, que además muestran una significativa alteración de su movimiento, siendo este más lento y desplazándose distancias más cortas.

Con esto, los resultados que hemos obtenido en este proyecto han dado lugar a las siguientes conclusiones:

1. Las larvas deficientes en Spint1a muestran un aumento en los niveles de estrés oxidativo y nitrosativo en la piel, promoviendo la inflamación crónica en piel, a su vez caracterizada por la infiltración de neutrófilos y la aparición de agregados de queratinocitos.
2. Los niveles de Gapdh se encuentran aumentados en la piel de las larvas deficientes en Spint1a y en las lesiones de pacientes humanos de psoriasis. Su translocación nuclear se ha asociado con la hiperproliferación y muerte celular de los queratinocitos. La inhibición farmacológica de GAPDH reduce la inflamación de la piel tanto en larvas de pez cebra deficientes en Spint1a como en modelos de psoriasis organotípicos humanos de piel en 3D.
3. El ARNm de *gapdh* se expresa específicamente en neutrófilos y células musculares. La inhibición genética y farmacológica de Gapdh alivia la inflamación en piel característica de la línea deficiente en Spint1a. Por su parte, *gapdhs* se expresa de forma general y su inhibición no afecta a la inflamación en piel.
4. Existe una correlación positiva entre el número de neutrófilos infiltrados en la piel y el número de agregados de queratinocitos en las larvas deficientes en Spint1a. Además, los neutrófilos empeoran la inflamación crónica en piel en este modelo.

5. La inhibición genética y farmacológica de la translocación de Gapdh al núcleo reduce el reclutamiento de neutrófilos, la velocidad y el tiempo de interacción con los agregados de queratinocitos en el modelo deficiente en Spint1a. Además, experimentos genéticos revelaron un papel de Gapdh sobre la biología del neutrófilo.
6. La translocación de Gapdh regula la expresión de varios genes, particularmente los asociados con inflamación, respuesta inmune y regulación génica en el modelo de pez cebra deficiente en Spint1a.
7. La inhibición farmacológica de la translocación de Gapdh al núcleo reduce la actividad NF-κB en la piel de la línea deficiente en Spint1a y alivia la inflamación en piel.
8. La translocación de Gapdh al núcleo induce la expresión de *il4* en neutrófilos en las larvas deficientes en Spint1a, y la Il4 producida por los neutrófilos incrementa la inflamación crónica en piel en este modelo.
9. La inhibición farmacológica de la translocación de Gapdh al núcleo reduce el reclutamiento de neutrófilos, su velocidad y desplazamiento hacia la cola cortada de las larvas de pez cebra.

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ANNEXE I: PUBLICATION CONTRIBUTION DURING THE PhD

Publications derived from the thesis

- **Martinez-Morcillo, F. J., J. Canton-Sandoval, F. J. Martinez-Navarro, I. Cabas, I. Martinez-Vicente, J. Armistead, J. Hatzold, A. Lopez-Munoz, T. Martinez-Menchon, R. Corbalan-Velez, J. Lacal, M. Hammerschmidt, J. C. Garcia-Borron, A. Garcia-Ayala, M. L. Cayuela, A. B. Perez-Oliva, D. Garcia-Moreno and V. Mulero (2021). "NAMPT-derived NAD+ fuels PARP1 to promote skin inflammation through parthanatos cell death." PLoS Biol 19(11): e3001455.**
- **Martinez-Morcillo, F. J., J. Canton-Sandoval, T. Martinez-Menchon, R. Corbalan-Velez, P. Mesa-Del-Castillo, A. B. Perez-Oliva, D. Garcia-Moreno and V. Mulero (2021). "Non-canonical roles of NAMPT and PARP in inflammation." Dev Comp Immunol 115: 103881.**

Other publications non-related to the thesis

- **Arroyo, A. B., M. Bernal-Carrion, J. Canton-Sandoval, I. Cabas, R. Corbalan-Velez, T. Martinez-Menchon, B. Ferri, M. L. Cayuela, D. Garcia-Moreno and V. Mulero (2023). "NAMPT and PARylation Are Involved in the Pathogenesis of Atopic Dermatitis." Int J Mol Sci 24(9).**
- **Camiolo, G., A. Barbato, C. Giallongo, N. Vicario, A. Romano, N. L. Parrinello, R. Parenti, J. C. Sandoval, D. Garcia-Moreno, G. Lazzarino, R. Avola, G. A. Palumbo, V. Mulero, G. Li Volti, D. Tibullo and F. Di Raimondo (2020). "Iron regulates myeloma cell/macrophage interaction and drives resistance to bortezomib." Redox Biol 36: 101611.**
- **Fatas-Lalana, B., J. Canton-Sandoval, L. Rodriguez-Ruiz, R. Corbalan-Velez, T. Martinez-Menchon, A. B. Perez-Oliva and V. Mulero (2022). "Impact of Comorbidities of Patients with Psoriasis on Phototherapy Responses." Int J Mol Sci 23(17).**
- **Hernandez-Silva, D., J. Canton-Sandoval, F. J. Martinez-Navarro, H. Perez-Sanchez, S. de Oliveira, V. Mulero, F. Alcaraz-Perez and M. L. Cayuela (2022). "Senescence-Independent Anti-Inflammatory Activity of the Senolytic Drugs Dasatinib, Navitoclax, and Venetoclax in Zebrafish Models of Chronic Inflammation." Int J Mol Sci 23(18).**

ANNEXE II: CONTRIBUTION TO SCIENTIFIC CONFERENCES DURING THE PhD

- **Joaquín Cantón Sandoval**; Francisco Javier Martínez Morcillo; Cassia Michael; Beatriz Bernal Bermúdez; Ana Belén Arroyo; Isabel Cabas; Alicia Martínez López; Annamaría Pedoto; María Luisa Cayuela; Sofía de Oliveira; Diana García Moreno; Victoriano Mulero. Inhibition of nuclear translocation of GAPDH impacts neutrophil migration and ameliorates chronic skin inflammation**.** European Phagocyte Workshop 2023, Budapest, Hungary. Oral Communication.
- **Joaquín Cantón Sandoval**; Francisco Javier Martínez Morcillo; Cassia Michael; Beatriz Bernal Bermúdez; Ana Belén Arroyo; Isabel Cabas; Alicia Martínez López; Annamaría Pedoto; María Luisa Cayuela; Sofía de Oliveira; Diana García Moreno; Victoriano Mulero. Inhibition of nuclear translocation of GAPDH impacts neutrophil migration and ameliorates chronic skin inflammation**.** VII Jornadas Doctorales. Universidad de Murcia, Murcia, Spain. Oral Communication.
- Annamaría Pedoto; **Joaquín Cantón Sandoval**; Sylwia Tyrkalska; Victoriano Mulero. Opposed roles of the inflammasome of neutrophils and macrophages in a zebrafish model of COVID-19-associated cytokine storm syndrome. European Phagocyte Workshop 2023, Budapest, Hungary. Oral Communication.
- **Joaquín Cantón Sandoval**; Francisco Javier Martínez Morcillo; Ana Belén Arroyo Rodríguez; Cassia Michael; Alicia Martínez López; Natalia Pérez Escudero; Beatriz Bernal Bermúdez; Martín Bernal Carrión; Isabel Cabas Sánchez; María Luisa Cayuela; Sofía de Oliveira; Diana García Moreno; Victoriano Mulero. Inhibition of nuclear translocation of GAPDH impacts neutrophil migration and ameliorates chronic skin inflammation. International Symposium Neutrophil 2022. México City, México. Oral Communication.
- Ana Belén Arroyo Rodríguez, Eva Bastida Martínez, Sylwia Tyrkalska, Antonio J Monera-Girona, **Joaquín Cantón Sandoval**, Martín Bernal Carrión, Diana García Moreno, Montserrat Elías Arnanz, Victoriano Mulero. Inhibition of plasmalogen synthesis exacerbates acute and chronic skin inflammation in zebrafish. 44 congreso Nacional de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM). Málaga, España. Oral Communication.
- Martín Bernal Carrión, Ana Belén Arroyo Rodríguez, **Joaquín Cantón Sandoval**, Natalia Pérez Escudero, Isabel Cabas, Raúl Corbalán Vélez, Teresa Martínez Menchón, Diana García Moreno, Victoriano Mulero. Role of melatonin in skin chronic inflammation. 44 congreso Nacional de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM). Málaga, España. Poster.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Ana Belén Arroyo Rodríguez, Beatriz Bernal Bermúdez, Isabel Cabas Sánchez, Natalia Pérez Escudero, Martín Bernal Carrión, Antonio Raúl Corbalán Pérez, María Teresa Martínez Menchón, María Luisa Cayuela, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero. INHIBITION OF NUCLEAR TRANSLOCATION OF GAPDH AMELIORATES CHRONIC SKIN INFLAMMATION. VI Jornadas Científicas del IMIB-Arrixaca. Hospital Virgen de la Arrixaca, Murcia, Spain. Poster.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Ana Belén Arroyo Rodríguez, Beatriz Bernal Bermúdez, Isabel Cabas Sánchez, Natalia Pérez Escudero, Martín Bernal Carrión, Antonio Raúl Corbalán Pérez, María Teresa Martínez Menchón, María Luisa Cayuela, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero. Oxidative stress fuels GAPDH nuclear translocation to promote skin inflammation

through PARP1 interaction. VI Jornadas Doctorales. Universidad de Murcia, Murcia, Spain. Oral Communication.

- Francisco J. Martinez-Morcillo, Alicia Martinez Lopez, Haleh Nikzamir, Isabel Cabas Sanchez, **Joaquin Canton-Sandoval**, Teresa Martinez-Menchon, Pablo Mesa del Castillo, Maria L Cayuela, Ana B. Perez-Oliva, Diana Garcia-Moreno, Victoriano Mulero. CHARACTERIZATION OF A NEW MUTATION CAUSING TYPE I INTERPHERONOPATHY AND DEVELOPMENT OF ANIMAL MODELS FOR PERSONALIZED MEDICINE. CRISPR-Cas: FROM MICROBIOLOGY TO BIOMEDICINE. Baeza, Andalucía, Spain. Oral Communication.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Francisco Juan Martínez Navarro, Idoya Mª Martínez Vicente, José Carlos García Borrón, Joy Armistead, Matthias Hammerschmidt, Isabel Cabas Sánchez, María Azucena López Muñoz, Antonio Raúl Corbalán Vélez, María Teresa Martínez Menchón, Alfonsa García Ayala, María Luisa Cayuela Fuentes, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero Méndez. Oxidative stress promotes PARP1 hyperactivation to induce skin inflammation through parthanatos. V Jornadas Científicas del IMIB-Arrixaca. Hospital Virgen de la Arrixaca, Murcia, Spain. Awarded Oral Communication.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Francisco Juan Martínez Navarro, Idoya Mª Martínez Vicente, José Carlos García Borrón, Joy Armistead, Matthias Hammerschmidt, Isabel Cabas Sánchez, María Azucena López Muñoz, Antonio Raúl Corbalán Vélez, María Teresa Martínez Menchón, Alfonsa García Ayala, María Luisa Cayuela Fuentes, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero Méndez. Oxidative stress promotes PARP1 hyperactivation to induce skin inflammation through parthanatos. V Jornadas Doctorales. Universidad de Murcia, Murcia, Spain. Oral Communication.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Francisco Juan Martínez Navarro, Idoya Mª Martínez Vicente, Antonio Raúl Corbalán Vélez, María Teresa Martínez Menchón, José Carlos García-Borrón Martínez, María Luisa Cayuela, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero. Modeling The Impact Of Oxidative Stress In Psoriasis. IV Jornadas Científicas del IMIB-Arrixaca. Hospital Virgen de la Arrixaca, Murcia, Spain. Oral Communication.
- **Joaquín Cantón Sandoval**, Francisco Javier Martínez Morcillo, Francisco Juan Martínez Navarro, Antonio Raúl Corbalán Vélez, María Teresa Martínez Menchón, María Luisa Cayuela, Ana Belén Pérez Oliva, Diana García Moreno, Victoriano Mulero. Modeling The Impact Of Oxidative Stress In Psoriasis. III Jornadas Científicas del IMIB-Arrixaca. Hospital Virgen de la Arrixaca, Murcia, Spain. Poster.

ANNEXE III: RESEARCH STAY IN OTHER LABORATORIES DURING THE PhD

ANNEXE III

Short stay at international research centre

Host institution: Albert Einstein College of Medicine

Country: New York, New York, United States of America

Responsible person in the Host: Dra. Sofia Ludovina Novais De Oliveira

Stay period: 20th June 2022 – 20th December 2022